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TESIS DOCTORAL

ESTUDIO TAXONÓMICO DE BACTERIAS RELACIONADAS CON EL SÍNDROME DEL ALEVÍN DE LA TRUCHA

MEMORIA PARA OPTAR AL GRADO DE DOCTORA

PRESENTADA POR

Leydis Zamora Morales

Directores

Ana Isabel Vela
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Lucas Domínguez

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Bajo la dirección de los doctores:

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CERTIFICAN:

Que la Tesis Doctoral Titulada "Estudio Taxonómico de Bacterias Relacionadas con el Síndrome del Alevín de la Trucha" ha sido realizada por la Licenciada en Microbiología Dª. Leydis Zamora Morales en el Servicio de Diagnóstico, Identificación y Caracterización Molecular del Centro de Vigilancia Sanitaria Veterinaria (VISAVET) de la Universidad Complutense de Madrid bajo la dirección conjunta de los que suscriben, y estiman que reúne los requisitos exigidos para optar al título de Doctor por la Universidad Complutense de Madrid.

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Los Directores de la Tesis Doctoral

Ana Isabel Vela Alonso José F. Fernández-Garayzábal Fernández Lucas Domínguez Rodríguez

A mis dos tesoros, David y Diego.

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Justificación

La presente Tesis Doctoral está constituida por un compendio de trabajos de investigación publicados durante el desarrollo de la misma en varias revistas científicas de carácter internacional. A continuación se presentan las referencias bibliográficas de cada una de las publicaciones que conforman esta Tesis:

Zamora L, Fernández-Garayzábal JF, Palacios MA, Sánchez-Porro C, Svensson-Stadler LA, Domínguez L, Moore ER, Ventosa A, Vela AI. (2012). *Chryseobacterium oncorhynchi* sp. nov., isolated from rainbow trout (*Oncorhynchus mykiss*). *Syst Appl Microbiol.*, 35, 24-29.

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Zamora L, Vela AI, Palacios MA, Sánchez-Porro C, Svensson-Stadler LA, Domínguez L, Moore ER, Ventosa A, Fernández-Garayzábal JF. (2012). *Chryseobacterium viscerum* sp. nov., isolated from diseased fish. *Int J Syst Evol Microbiol.*, 62, 2934-2940.

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Zamora L, Vela AI, Palacios MA, Sánchez-Porro C, Moore ER, Domínguez L, Ventosa A, Fernández-Garayzábal JF. (2012). *Chryseobacterium tructae* sp. nov., isolated from rainbow trout (*Oncorhynchus mykiss*). *Syst Appl Microbiol.*, 35, 315-319.

Zamora L, Fernández-Garayzábal JF, Sánchez-Porro C, Palacios MA, Moore ER, Domínguez L, Ventosa A, Vela AI. (2013). *Flavobacterium plurextorum* sp. nov. Isolated from Farmed Rainbow Trout (*Oncorhynchus mykiss*). *PLoS One*., 8, 67741. doi: 10.1371/journal.pone.0067741.

Zamora L, Vela AI, Sánchez-Porro C, Palacios MA, Domínguez L, Moore ER, Ventosa A, Fernández-Garayzábal JF. (2013). Characterization of flavobacteria possibly associated with fish and fish farm environment. Description of three novel *Flavobacterium* species: *Flavobacterium collinsii* sp. nov., *Flavobacterium branchiarum* sp. nov., and *Flavobacterium branchiicola* sp. nov. *Aquaculture.,* 416-417, 346-353.

Zamora L, Vela AI, Sánchez-Porro C, Palacios MA, Moore ER, Domínguez L, Ventosa A, Fernández-Garayzábal JF. (2014). *Flavobacterium tructae* sp. nov. and *Flavobacterium piscis* sp. nov., isolated from farmed rainbow trout (*Oncorhynchus mykiss*). *Int J Syst Evol Microbiol.*, 64, 392-399.

Índice

Resumen

1 Resumen

Los géneros *Flavobacterium* y *Chryseobacterium* pertenecen a la familia *Flavobacteriaceae* y están formados por microorganismos muy ubicuos que se aíslan, entre otras muchas fuentes, de ambientes acuáticos (Bernardet y Bowman, 2006; Bernardet y col., 2006). Aunque la mayoría de las especies incluidas en ambos géneros son saprofitas, algunas especies como *F. branchiophilum*, *F. columnare* y *F. psychrophilum* son reconocidas como importantes patógenos de peces (Ostland y col., 1994; Barnes y Brown, 2011; Declercq y col., 2013) y otras, como *C. scophthalmum*, *C. piscicola*, *C. piscium*, *C. chaponense*, *F. johnsoniae*, *F. hydatis*, *F. succinicans*, *F. chilense, F. araucananum* y *F. spartansii,* se han aislado de peces enfermos (Mudarris y col., 1994; Bernardet y Bowman, 2006; De Beer y col., 2006; Flemming y col., 2007; Ilardi y col., 2009; Kämpfer y col., 2011, 2012; Loch y Faisal, 2014b).

En el presente trabajo de Tesis Doctoral hemos realizado un estudio taxonómico polifásico de un grupo de bacterias Gram negativas aisladas de alevines enfermos de trucha arcoíris que presentaban síntomas clínicos compatibles con un proceso septicémico causado por *F. psychrophilum* (Austin y Stobie, 1991), así como de varios aislados obtenidos de huevos embrionados. Debido a que estos microorganismos fueron recuperados mayoritariamente de alevines enfermos y en algunos casos se obtuvieron en cultivo puro, consideramos que podrían tener una significación clínica en el proceso infeccioso que padecían los alevines y por ello decidimos realizar un estudio exhaustivo de los mismos con la finalidad de poder identificarlos correctamente.

Los aislados se obtuvieron en agar *Anacker and Ordal*, uno de los medios de cultivo empleados comúnmente para el aislamiento de *F. psychrophilum* (Anacker y Ordal, 1959). Sin embargo, tras la tinción de Gram, ninguno de los microorganismos aislados mostró la típica morfología de bacilos filamentosos que exhibe *F. psychrophilum* (Cipriano y Holt, 2005) y tampoco fue posible la identificación de estos microorganismos mediante la utilización de una PCR específica para este patógeno (Wiklund y col., 2000).

Teniendo en cuenta el color de las colonias los aislados se dividieron en dos grupos. Un grupo crecía formando colonias de color amarillo, mientras que el otro formaba colonias de color naranja. La primera aproximación a la identificación de estos microorganismos la realizamos mediante la

identificación molecular basada en la secuenciación del gen 16S ARNr, lo que permitió la adscripción de los mismos a los géneros *Flavobacterium* y *Chryseobacterium*. Los aislados que formaron colonias amarillas constituyeron siete grupos filogenéticos (F1-F7) que mostraron porcentajes de similitud en la secuencia de dicho gen mayores del 98,9% con diferentes especies del género *Flavobacterium*, mientras que los aislados que formaron colonias naranja se agruparon formando cuatro grupos filogenéticos (C1-C4) que presentaron porcentajes de similitud en la secuencia del gen 16S ARNr mayores del 99,1% con distintas especies del género *Chryseobacterium*.

Los aislados del grupo C4 se relacionaron filogenéticamente con *C. shigense*. Estos aislados presentaron una morfología macroscópica y microscópica idéntica a la de la cepa tipo de *C. shigense* (GUM-Kaji^T; Shimomura y col., 2005), así como unas características fenotípicas consistentes con las indicadas en la descripción actual de esta especie (Shimomura y col., 2005). Basándonos en los datos de secuenciación del gen 16S ARNr, y los resultados de la caracterización fenotípica, los aislados de este grupo se identificaron como *C. shigense*. Esta especie fue aislada originalmente de una bebida de ácido láctico (Shimomura y col., 2005) y no hay publicaciones previas que relacionen a este microorganismo con peces, siendo esta la primera vez que se aísla a partir de peces enfermos.

Teniendo en cuenta que en los géneros *Flavobacterium* y *Chryseobacterium* especies diferentes pueden presentar homologías de hasta 99% en las secuencias del 16S ARNr (Bernardet, 2006; Charimba y col., 2013; Subhash y col., 2013; Kämpfer y col., 2014a, b; Loch y Faisal, 2014b), realizamos, en los grupos F1-F7 y C1-C3, estudios de hibridación ADN-ADN con las especies filogenéticamente más próximas. Los resultados mostraron porcentajes de hibridación que oscilaron entre 2 y 59,4% con las especies filogenéticamente más próximas a cada grupo, valores claramente inferiores al porcentaje del 70% de homología considerado como mínimo para asignar dos taxones a la misma especie (Wayne y col., 1987).

Los resultados obtenidos en el estudio filogenético y de hibridación ADN-ADN permitieron demostrar que los microorganismos de los grupos F1-F7 y C1-C3 se correspondían con siete nuevas especies dentro del género *Flavobacterium* y tres nuevas especies dentro del género *Chryseobacterium* para las cuales se propusieron los nombres específicos de *Flavobacterium oncorhynchi* sp. nov. (grupo F1), *Flavobacterium plurextorum* sp. nov. (grupo

F2), *Flavobacterium tructae* sp. nov. (grupo F3), *Flavobacterium piscis* sp. nov. (grupo F4), *Flavobacterium collinsii* sp. nov. (grupo F5), *Flavobacterium branchiarum* sp. nov. (grupo F6), *Flavobacterium branchiicola* sp. nov. (grupo F7), *Chryseobacterium oncorhynchi* sp. nov. (grupo C1), *Chryseobacterium tructae* sp. nov. (grupo C2) y *Chriseobacterium viscerum* sp. nov. (grupo C3).

Con el fin de poder realizar la descripción formal de las nuevas especies se completaron los estudios filogenéticos y de hibridación ADN-ADN con la determinación del contenido en G+C del ADN y la caracterización quimiotaxonómica y fenotípica de los aislados.

El contenido en G+C del ADN varió para las especies del género *Flavobacterium* entre 33,0 y 36,2 mol% y para las del género *Chryseobacterium* entre 33,1 y 39,0 mol%, valores que están dentro del rango descrito para ambos géneros (Bernardet y Bowman, 2006; Bernardet y col., 2006).

El estudio de las características quimiotaxonómicas mostró que todas las especies presentaban MK-6 como principal quinona respiratoria y en algunas especies (*F. plurextorum*, *F. tructae*, *F. piscis, F. collinsii, F. branchiarum, F. branchiicola* y *C. oncorhynchi*) también se encontraron pequeñas cantidades de MK-5. En relación a los diferentes ácidos grasos, aunque existieron diferencias cuantitativas en el porcentaje de cada uno de los ácidos grados detectados entre las diferentes especies, todas ellas mostraron iso-$C_{15:0}$ como ácido graso mayoritario, observándose niveles de entre 19-33% para las nuevas especies del género *Flavobacterium* y en torno al 33,7-40,9% para las del género *Chryseobacterium*. Respecto a los lípidos polares, esta característica fue estudiada en las especies *F. oncorhynchi*, *F. tructae*, *F. piscis*, *F. collinsii*, *F. branchiarum* y *F. branchiicola*, las cuales presentaron fosfatidiletanolamina como principal lípido polar, aunque también se encontraron varios aminolípidos y lípidos no identificados. Todos los resultados quimiotaxonómicos obtenidos coinciden con los descritos para los miembros de los géneros *Flavobacterium* y *Chryseobacterium* (Vandamme y col., 1994a; Bernardet y col., 2002; Bernardet y Nakagawa, 2006; Bernardet y Bowman, 2011) y confirman la adscripción de las nuevas especies a estos géneros basada en los estudios filogenéticos.

Las nuevas especies de los géneros *Flavobacterium* y *Chryseobacterium* pueden diferenciarse entre sí por diferentes características fenotípicas. Así por ejemplo, las nuevas especies del género *Flavobacterium* se diferencian en

pruebas como la degradación de la L-tirosina (positiva para las especies *F. plurextorum*, *F. tructae*, *F. collinsii* y *F. branchiicola* y negativa para *F. oncorhynchi*, *F. piscis* y *F. branchiarum*), la degradación de la urea (únicamente positiva para *F. branchiicola*), la formación de pigmento difusible marrón en agar L-tirosina (observado solo en la especie *F. oncorhynchi*) y la actividad β-galactosidasa (ausente solamente en *F. branchiarum*). De igual forma, las nuevas especies del género *Chryseobacterium* pueden diferenciarse por pruebas como el crecimiento en medio BHI con 3% de NaCl (observado solamente en la especie *C. tructae*), la utilización de D-manitol como fuente de carbono y energía y la presencia de actividad N-acetil-beta-glucosaminidasa (ambas positivas únicamente para *C. viscerum*), la presencia de las enzimas tripsina y α-glucosidasa en *C. viscerum* y *C. oncorhynchi,* y la ausencia de actividad fosfoamidasa en *C. oncorhynchi.*

Las nuevas especies descritas y los aislados de *C. shigense* fueron caracterizados molecularmente mediante la técnica PFGE y la técnica RAPD, respectivamente. Los resultados mostraron una posible significación clínica de algunos de estos microorganismos. Por ejemplo en las especies *F. oncorhynchi* y *C. oncorhynchi* aislados recuperados de alevines diferentes y procedentes de diferentes episodios clínicos ocurridos en diferentes periodos de tiempo, mostraron patrones indistinguibles de PFGE indicando que se trataban de una misma cepa. Algo similar ocurrió con los aislados de *C. shigense* cuando se tipificaron mediante la técnica RAPD, que mostraron un único perfil molecular a pesar de haber sido aislados durante cinco episodios clínicos diferentes. Sin embargo, el papel de estas especies como patógenos de peces deberá confirmarse mediante ensayos exhaustivos de infecciones experimentales.

Los resultados obtenidos en este estudio no solo han permitido el reconocimiento y la descripción de diez nuevas especies de los géneros *Flavobacterium* y *Chryseobacterium* y el aislamiento por primera vez de la especie *C. shigense* a partir peces enfermos, sino que además ayudan a tener un mejor conocimiento de la diversidad de especies de ambos géneros relacionadas con infecciones en peces. Estos resultados junto al aislamiento creciente de especies de ambos géneros a partir de diferentes muestras clínicas de peces (Pacha y Porter, 1968; Rintamaki-Kinnunen y col., 1997; Tiirola y col., 2002; Bernardet y col., 2005; Flemming y col., 2007; Ilardi y Avendaño-Herrera, 2008; Loch y col., 2013) ponen de manifiesto el papel de estos microorganismos como potenciales patógenos de peces. En este trabajo

se describen además una serie de pruebas que pueden ayudar a la correcta identificación de las distintas especies de los géneros *Flavobacterium* y *Chryseobacterium* asociadas a procesos clínicos en peces.

Summary

2 Summary

The genera *Flavobacterium* and *Chryseobacterium* belong to the family *Flavobacteriaceae.* Both genera are formed by ubiquitous microorganisms, which can be isolated from a wide range of environments including aquatic environments (Bernardet *et al*., 2006; Bernardet and Bowman, 2006). Although most species of both genera are saprophytes, some species such as *F. branchiophilum*, *F. columnare* and *F. psychrophilum* are important fish pathogens (Ostland *et al*., 1994; Barnes and Brown, 2011; Declercq *et al*., 2013). Several other species, such as *C. scophthalmum*, *C. piscicola*, *C. piscium*, *C. chaponense*, *F. johnsoniae*, *F. hydatis*, *F. succinicans*, *F. chilense, F. araucananum and F. spartansii,* have also been occasionally associated with infections in fish (Mudarris *et al*., 1994; Bernardet and Bowman, 2006; De Beer *et al*., 2006; Flemming *et al*., 2007; Ilardi *et al*., 2009; Kämpfer *et al*., 2011, 2012; Lochy and Faisal, 2014b).

In the present work a group of Gram-negative bacteria isolated from eggs and diseased juvenile rainbow trout suspected of *F. psychrophilum* infection (Austin and Stobie, 1991), were studied using a taxonomic polyphasic approach. Many of these isolates were obtained from diseased rainbow fry, sometimes in pure culture, which might be indicative of their implication in the infection affecting rainbow trout fry. Therefore, we decided to undertake a comprehensive study in order to reach their precise identification.

Isolates were recovered on Anacker and Ordal agar, medium commonly used for the isolation of *F. psychrophilum* (Anacker and Ordal, 1959). However, the cell morphology of the trout isolates following Gram staining was different of the filamentous rods exhibited by *F. psychrophilum* (Cipriano and Holt, 2005) and none gave a positive reaction with a *F. psychrophilum* species-specific PCR assay (Wiklund *et al*., 2000).

Isolates were separated into two groups based on the color displayed by their colonies. One group included isolates displaying yellow colonies, while the other consisted of isolates giving orange colonies. Preliminary identification of the isolates was carry out by sequencing of their 16S rRNA gene. Analysis of 16S rRNA sequences of these isolates revealed a clear affiliation to the genera *Flavobacterium* and *Chryseobacterium*. Isolates displaying yellow colonies represented seven separate phylogenetic groups (F1-F7) most closely related (sequence similarities higher than 98.9%) to

different members of the genus *Flavobacterium.* The isolates displaying orange colonies represented four separate phylogenetic groups (C1-C4) with sequence similarities higher than 99.1% to the members of the genus *Chryseobacterium.*

Isolates of the phylogenetic group C4 exhibited the highest 16S rRNA sequence similarities with *C. shigense*. These isolates exhibited a macroscopic and microscopic morphology identical to the type strain of *C. shigense* (Shimomura *et al*., 2005). Moreover, most of the phenotypic characteristics of the *C. shigense* trout isolates were consistent with the current description of this species based solely in the strain DSM 17126^{T} (Shimomura *et al*., 2005), which support the identification based on 16S rRNA gene sequencing. Taking into account the results of 16S rRNA gene sequencing and the results of the phenotypic characterization, C4 group isolates were identified as *C. shigense*. This is the first description of the recovery of *C. shigense* from clinical specimens in trout, a very different habitat to fresh lactic acid beverage where it was initially isolated (Shimomura *et al*., 2005).

Within the genera *Flavobacterium* and *Chryseobacterium,* different species can exhibit up to 99.0% similarity in their 16S rRNA gene sequences (Bernardet, 2006; Charimba *et al*., 2013; Subhash *et al*., 2013; Kämpfer *et al*., 2014a, b; Loch and Faisal, 2014b). For that reason, genomic DNA–DNA hybridizations were carried out between the isolates of F1-F7 and C1-C3 groups and their respective closet phylogenetically related species. DNA–DNA hybridizations yielded reassociation values between 2-59.4% with the closest phylogenetically species of each group. These values were below the 70% cut-off point for species delineation (Wayne *et al*., 1987).

The phylogenetic and genomic DNA–DNA hybridizations data demonstrated that the isolates of F1-F7 and C1-C3 groups represent seven and three new species belonging to the genera *Flavobacterium* and *Chryseobacterium*, respectively. The names *Flavobacterium oncorhynchi* sp. nov. (group F1), *Flavobacterium plurextorum* sp. nov. (group F2), *Flavobacterium tructae* sp. nov. (group F3), *Flavobacterium piscis* sp. nov. (group F4)*, Flavobacterium collinsii* sp. nov. (group F5)*, Flavobacterium branchiarum* sp. nov. (group F6) and *Flavobacterium branchiicola* sp. nov. (group F7) are proposed for the new species of the genus *Flavobacterium.* On the other hand, the names *Chryseobacterium oncorhynchi* sp. nov. (group C1), *Chryseobacterium tructae* sp. nov. (group C2) and *Chriseobacterium viscerum*

sp. nov. (group C3) are proposed for the new species of the genus *Chryseobacterium*.

In order to describe formally the new species, the G+C content of the DNA of a representative isolate of each group was determined. In addition, the chemotaxonomic, physiological and biochemical characteristics of isolates were determined.

The DNA G+C content was between 33.0-36.2 mol% and 33.1-39.0 mol% for the species of the genus *Flavobacterium* and *Chryseobacterium*, respectively. These values are consistent with what has been observed for species of both genera (Bernadet *et al*., 2006; Bernardet and Bowman, 2006).

Menaquinone-6 (MK-6) was the major respiratory quinone in all species, in line with all members of the family *Flavobacteriaceae*. Some species also contained minor amounts of MK-5 (*F. plurextorum*, *F. tructae*, *F. piscis, F. collinsii, F. branchiarum, F. branchiicola* and *C. oncorhynchi*). Analysis of whole-cell fatty acids revealed that iso-C15:0 was the predominant cell fatty acid in all species, with percentages that ranged between 19.0-33.0% and 3.7-40.9% for the new species of the genera *Flavobacterium* and *Chryseobacterium*, respectively. The polar lipids were determined in the species *F. oncorhynchi*, *F. tructae*, *F. piscis*, *F. collinsii*, *F. branchiarum* and *F. branchiicola*, which presented phosphatidylethanolamine as the main polar lipid, although several unidentified aminolipids and unidentified lipids were also found. These chemotaxonomic data corroborated the adscription of the novel species to the genera *Flavobacterium* and *Chryseobacterium* (Vandamme *et al*., 1994a; Bernardet *et al*., 2002; Bernardet and Nakagawa, 2006; Bernardet and Bowman, 2011).

The new species of the genera *Flavobacterium* and *Chryseobacterium* can be readily differentiated by several morphological, physiological and biochemical characteristics. For example, the new species of the genus *Flavobacterium* can be differentiated by their ability to degrade L-tyrosine (positive for *F. plurextorum*, *F. tructae*, *F. collinsii* and *F. branchiicola* and negative for *F. oncorhynchi*, *F. piscis* and *F. branchiarum*) and urea (only positive for *F. branchiicola*). The can be also distinguished by the production of brown diffusible pigment on tyrosine agar (observed only in the specie *F. oncorhynchi*) or β-galactosidase activity (absent only in *F. branchiarum*). Similarly, the new species of the genus *Chryseobacterium* can be differentiated

by their ability to grow in brain heart infusion broth with 3% added NaCl (observed only in *C. tructae*), the use of D-mannitol as sole carbon and energy sources and the detection of N-acetyl-β-glucosaminidase activity (both positive only for *C. viscerum*), the presence of the enzymes trypsin and α-glucosidase in *C. viscerum* and *C. oncorhynchi*, and absence of phosphoamidase activity in *C. oncorhynchi.*

Isolates of the new species described and *C. shigense* isolates were molecularly characterized by PFGE and RAPD, respectively. The results suggested a possible clinical significance of some of these microorganisms. For instance, isolates of *F. oncorhynchi* and *C. oncorhynchi*, obtained from different rainbow trout fry in several clinical episodes at different times, exhibited indistinguishable PFGE patterns indicating they are the same strain. Similarly, *C. shigense* isolates showed undistinguishable RAPD fingerprints although they had been isolated for five different clinical episodes. However, the role of these species as fish pathogens should be confirmed by experimental infection trials.

The results obtained in this study allowed the identification and description of ten new species belonging to the genera *Flavobacterium* and *Chryseobacterium* and the first description of the isolation of *C. shigense* from clinical specimens in fish. In addition, the results of the present work contribute to a better knowledge of the diversity of species of both genera from fish and their possible association with disease. On the other hand, these results, together with the increasing number of species of both genera isolated from different clinical specimens of fish (Pacha and Porter, 1968; Rintamaki-Kinnunen *et al.*, 1997; Tiirola *et al*., 2002; Bernardet *et al*., 2005; Fleming *et al*., 2007; Ilardi and Avendaño-Herrera, 2008;. Loch *et al.*, 2013) highlight the role of these microorganisms as potential fish pathogens. In addition, we provide different characteristics that can facilitate the correct identification of the different *Flavobacterium* and *Chryseobacterium* species currently associated with fish disease or isolated from diseased fish.

Introducción

3 Introducción

3.1 Familia *Flavobacteriaceae*

3.1.1 Aspectos taxonómicos

La familia *Flavobacteriaceae* pertenece al Orden *Flavobacteriales* y a la Clase *Flavobacteriia*, y representa la mayor rama del filo *Bacteroidetes*, anteriormente denominado grupo *Cytophaga-Flavobacterium-Bacteroides* (Hirsch y col., 1998). La primera alusión a esta familia la hizo Jooste en su estudio de Tesis Doctoral (Jooste, 1985). Posteriormente, la familia fue mencionada brevemente por Reichenbach (1989) en la primera edición del *Bergey's Manual of Systematic Bacteriology*, aunque no quedó descrita formalmente hasta 1992 cuando se validó el nombre de la familia (Reichenbach, 1992).

La primera descripción amplia de la familia se publicó en el año 1996 por Bernardet y col. quedando constituida por siete géneros (el género tipo, *Flavobacterium*, y los géneros *Bergeyella*, *Capnocytophaga*, *Chryseobacterium*, *Empedobacter*, *Ornithobacterium* y *Weeksella*), el taxón [*Flavobacterium*] *odoratum*, y algunos microorganismos aislados de ambientes marinos ([*Flexibacter*] *aurantiacus* subsp. *copepodarum*, [*Flexibacter*] *maritimus*, [*Flexibacter*] *ovolyticus*, [*Flectobacillus*] *glomeratus*, [*Cytophaga*] *latercula*, [*Cytophaga*] *lytica*, [*Cytophaga*] *marinoflava*, [*Cytophaga*] *uliginosa*, [*Flavobacterium*] *gondwanense* y [*Flavobacterium*] *salegens*).

Posteriormente, se sumaron a la familia varios nuevos géneros quedando reclasificados en ellos la mayoría de los microorganismos marinos agrupados en la familia y que no estaban adscritos a ninguno de los géneros que la formaban (ver párrafo anterior). Así, se creó el género *Myroides*, al que fue trasladado el taxón [*F.*] *odoratum* (Vancanneyt y col., 1996) y se describieron cinco géneros de microorganismos aislados de ambientes polares: *Gelidibacter* y *Psychroserpens* (Bowman y col., 1997), *Polaribacter* (incluyó la especie anteriormente llamada [*F.*] *glomeratus*) (Gosink y col., 1998), *Psychroflexus* (incluyó la especie anteriormente llamada [*F.*] *gondwanense*) (Bowman y col., 1998), y *Salegentibacter* (formado por la especie [*F.*] *salegens*) (McCammon y Bowman, 2000). Además, se describieron los géneros *Cellulophaga* (incluyó la especie anteriormente llamada [*C.*] *lytica*) (Johansen y col., 1999), *Tenacibaculum* (incluyó las especies anteriormente

llamadas [*F.*] *maritimus* y [*F.*] *ovolyticus*) (Suzuki y col., 2001) y *Zobellia* (incluyó la especie anteriormente denominada [*C.*] *uliginosa*) (Barbeyron y col., 2001).

En el año 2002 se publicaron los criterios mínimos (*Minimal Standards*) recomendados para la descripción de nuevos miembros de la familia *Flavobacteriaceae* (Bernardet y col., 2002) con el objetivo de facilitar la correcta descripción de nuevos taxones en la familia, y evitar así la publicación de nuevas especies pobremente caracterizadas (Tabla 1). Junto a estos criterios, se realizó también una modificación de la descripción de la familia, la cual era necesaria debido a la incorporación de nuevos taxones desde su primera descripción en 1996 que presentaban características distintas a las incluidas en la descripción original de la familia, quedando formada por 18 géneros y dos organismos genéricamente mal clasificados, las especies [*C.*] *marinoflava* y [*C.*] *latercula* (Bernardet y col., 2002). Estas dos últimas pasaron un poco más tarde a formar parte de los géneros *Leeuwenhoekiella* (Nedashkovskaya y col., 2005b) y *Aquimarina* (Nedashkovskaya y col., 2005a, 2006), respectivamente.

Tabla 1. *Minimal Standards* recomendados para la descripción de nuevos géneros en la familia *Flavobacteriaceae*

Características	**Propiedades observadas**
Fenotípicas	Producción de pigmento; Presencia de *Gliding motility*; Requerimientos de salinidad; Metabolismo capnofílico; Crecimiento a diferentes temperaturas (25, 37 y 42 °C); Crecimiento en agar MacConkey y hridroxi-β-butirato; Producción de ácido a partir de glucosa y sacarosa; Producción de DNasa, ureasa, catalasa, indol y β-galactosidasa; Degradación de agar, almidón, esculina y gelatina; Resistencia a la penicilina G.
Quimiotaxonómicas	Análisis de ácidos grasos; Menaquinonas
Genéticas	Contenido de G+C; Hibridación ADN-ADN; Secuenciación del 16S ARNr.

A partir de ese momento, el perfeccionamiento y mayor accesibilidad de las técnicas moleculares, unido a un aumento en el número de estudios de comunidades bacterianas en diversos ecosistemas, principalmente ambientes polares y/o marinos, permitió que el número de géneros atribuidos a la familia comenzara a crecer a un ritmo exponencial (Bernardet, 2006). Así, en el momento de escribir esta memoria (octubre de 2014), la familia *Flavobacteriaceae* está constituida por un total de 111 géneros cuyos nombres se muestran en la Tabla 2.

Tabla 2. Relación de géneros incluidos en la familia *Flavobacteriaceae*

Actibacter	*Aequorivita*	*Aestuariibaculum*	*Aestuariicola*
Algibacter	*Aquimarina*	*Arenibacter*	*Aureicoccus*
Aureitalea	*Aureivirga*	*Bergeyella*	*Bizionia*
Capnocytophaga	*Cellulophaga*	*Chryseobacterium*	*Cloacibacterium*
Coenonia	*Corallibacter*	*Costertonia*	*Croceibacter*
Croceitalea	*Cruoricaptor*	*Dokdonia*	*Elizabethkingia*
Empedobacter	*Epilithonimonas*	*Eudoraea*	*Euzebyella*
Flagellimonas	*Flaviramulus*	*Flavivirga*	*Flavobacterium*
Formosa	*Fulvibacter*	*Gaetbulibacter*	*Galbibacter*
Gangjinia	*Gelidibacter*	*Gillisia*	*Gilvibacter*
Gramella	*Hyunsoonleella*	*Imtechella*	*Jejuia*
Joostella	*Kordia*	*Kriegella*	*Krokinobacter*
Lacinutrix	*Leeuwenhoekiella*	*Leptobacterium*	*Lutaonella*
Lutibacter	*Lutimonas*	*Mangrovimonas*	*Maribacter*
Mariniflexile	*Marinivirga*	*Maritimimonas*	*Marixanthomonas*
Meridianimaribacter	*Mesoflavibacter*	*Mesonia*	*Muricauda*
Muriicola	*Myroides*	*Namhaeicola*	*Nonlabens*
Olleya	*Ornithobacterium*	*Persicivirga*	*Pibocella*
Planobacterium	*Polaribacter*	*Pontirhabdus*	*Postechiella*
Pricia	*Pseudofulvibacter*	*Pseudozobellia*	*Psychroflexus*
Psychroserpens	*Riemerella*	*Robiginitalea*	*Salegentibacter*
Salinimicrobium	*Sandarakinotalea*	*Sediminibacter*	*Sediminicola*
Sejongia	*Siansivirga*	*Sinomicrobium*	*Snuella*
Soonwooa	*Spongiibacterium*	*Stanierella*	*Stenothermobacter*
Subsaxibacter	*Subsaximicrobium*	*Sungkyunkwania*	*Tamlana*
Tenacibaculum	*Ulvibacter*	*Vitellibacter*	*Wautersiella*
Weeksella	*Winogradskyella*	*Yeosuana*	*Zeaxanthinibacter*
Zhouia	*Zobellia*	*Zunongwangia*	

http://www.bacterio.net/-classifgenerafamilies.html#Flavobacteriaceae

Desde el punto de vista filogenético, la familia *Flavobacteriaceae* ha sido analizada por varios autores a través de la comparación de secuencias de los genes que codifican para el 16S ARNr y la subunidad B de la ADN-girasa (*gyrB*) (Suzuki y col., 2001; Bernardet y col., 2002; Bernardet, 2006; Bernardet y Nakagawa, 2006). En el estudio más reciente (Bernardet, 2006), se compararon las secuencias del 16S ARNr de las especies representativas de los 54 géneros que en ese momento conformaban la familia, observándose que ésta se dividía en dos grupos bien definidos: uno más pequeño, que al parecer agrupaba géneros que incluían microorganismos desprovistos de *gliding motility*, incapaces de crecer bajo condiciones halófilas (excepto algunas especies del género *Chryseobacterium*) o psicrófilas (excepto los miembros del género *Sejongia*) y muchos de los miembros no pigmentados y patógenos de la familia; el otro grupo, más numeroso, también llamado "grupo marino" (Bowman y Nichols, 2005; Bowman, 2006), estaba formado mayoritariamente por géneros que abarcaban microorganismos halotolerantes o halófilos, aproximadamente la mitad de los cuales presentaban *gliding motility* y una tercera parte eran psicrófilos. Estos dos grupos basados en la comparación de las secuencias del gen que codifica para el 16S ARNr se mantienen actualmente con los 111 géneros que en la actualidad conforman esta familia (Figura 1).

Figura 1. Relaciones filogenéticas entre los representantes de los géneros de la familia *Flavobacteriaceae* basadas en la comparación de las secuencias del gen 16S ARNr.

3.1.2 Características fenotípicas, genotípicas y quimiotaxonómicas

Según la descripción de la familia *Flavobacteriaceae* (Bernardet y col., 2002), los microorganismos que la constituyen son bacilos cortos a moderadamente largos con lados paralelos o ligeramente irregulares y extremos redondeados o ligeramente cónicos. Por lo general miden de 0,3 a 0,6 µm de ancho y de 1 a 10 µm de largo, aunque los miembros de algunas especies, bajo determinadas condiciones de crecimiento, pueden formar células filamentosas flexibles (por ejemplo en los géneros *Flavobacterium* y *Tenacibaculum*) o células en espiral y helicoidal (especies de los géneros *Polaribacter*, *Psychroflexus* y *Psychroserpens*). En algunas especies las células de cultivos viejos pueden formar cuerpos esféricos o cocoides (por ejemplo en los géneros *Flavobacterium*, *Psychroserpens* y *Tenacibaculum*). Son bacterias Gram-negativas, que no forman esporos. Generalmente no presentan flagelos, a excepción de *Polaribacter irgensii*, que presenta flagelos polares. Pueden ser móviles por *gliding* (por ejemplo los géneros *Capnocytophaga*, *Cellulophaga*, *Gelidibacter*, *Flavobacterium*, *Tenacibaculum* y *Zobellia*) o inmóviles (por ejemplo los géneros *Bergeyella*, *Chryseobacterium*, *Coenonia*, *Empedobacter*, *Myroides*, *Ornithobacterium*, *Polaribacter*, *Psychroserpens*, *Riemerella*, *Salegentibacter* y *Weeksella*).

Estos microorganismos pueden presentar un crecimiento aerobio (por ejemplo los géneros *Bergeyella*, *Cellulophaga*, *Chryseobacterium*, *Empedobacter*, *Flavobacterium*, *Gelidibacter*, *Myroides*, *Polaribacter*, *Psychroflexus*, *Psychroserpens*, *Salegentibacter*, *Tenacibaculum*, *Weeksella* y *Zobellia*) o microaerófilo o anaerobio (por ejemplo los géneros *Capnocytophaga*, *Coenonia*, *Ornithobacterium* y *Riemerella*). La temperatura óptima de crecimiento oscila generalmente en el rango de 25 a 35 °C, si bien algunas especies o géneros son psicrófilos o psicrotolerantes (por ejemplo *Flavobacterium psychrophilum* y algunas especies de los géneros *Gelidibacter*, *Polaribacter*, *Psychroflexus* y *Psychroserpens*. Los miembros de algunos géneros son halófilos en diversos grados (por ejemplo en los géneros *Cellulophaga*, *Gelidibacter*, *Polaribacter*, *Psychroflexus*, *Psychroserpens*, *Salegentibacter*, *Tenacibaculum* y *Zobellia*).

Forman colonias que pueden ser no pigmentadas (por ejemplo en los géneros *Bergeyella*, *Coenonia*, *Ornithobacterium* y *Weeksella*), o pigmentadas debido a la producción de pigmentos del tipo flexirrubina, carotenoide o ambos (por ejemplo en los géneros *Capnocytophaga*, *Cellulophaga*, *Chryseobacterium*,

Empedobacter, *Flavobacterium*, *Gelidibacter*, *Myroides, Polaribacter*, *Psychroflexus*, *Psychroserpens*, *Riemerella*, *Salegentibacter*, *Tenacibaculum* y *Zobellia*). Son quimioorganotrofos. No presentan gránulos intracelulares de poly-β-hidroxibutirato. El contenido G+C del ADN varía de 27 a 44 mol%.

Desde el punto de vista quimiotaxonómico, todos los miembros de la familia contienen menaquinona 6 (MK-6) como única o principal quinona respiratoria y no poseen esfingofosfolípidos. Como poliamina mayoritaria los miembros de esta familia tienen homoespermidina, si bien también pueden presentar con frecuencia agmatina, cadaverina y putrescina como componentes minoritarios. Los ácidos grasos predominantes que se encuentran en los miembros de esta familia son generalmente característicos de géneros, aunque, siempre que se utilicen condiciones estandarizadas de cultivo, algunos perfiles de ácidos grasos ayudan a diferenciar especies (Vandamme y col., 1994b; Bernardet y col., 1996; Vancanneyt y col., 1996; Vandamme y col., 1996; Bowman y col., 1997, 1998; Gosink y col., 1998; Barbeyron y col., 2001). Los perfiles de proteínas también pueden ser útiles para diferenciar las especies de algunos géneros, como es el caso de los géneros *Capnocytophaga* (Vandamme y col., 1996), *Myroides* (Vancanneyt y col., 1996), *Riemerella* (Vancanneyt y col., 1999) o *Flavobacterium* (Bernardet y col., 1996).

3.1.3 Ecología

Los miembros de la familia *Flavobacteriaceae* son microorganismos muy ubicuos que habitan una amplia variedad de hábitats terrestres y acuáticos. La mayoría son saprófitos, aunque varios miembros de la familia se han aislado de personas y animales enfermos y algunas especies son consideradas verdaderos agentes patógenos (Bernardet y col., 1996, 2002).

Teniendo en cuenta el gran número de taxones incluidos en la familia *Flavobacteriaceae* (Euzeby y Parte, 2014b), las fuentes de las que se aíslan estos microorganismos son muy diversas, entre estas se pueden mencionar: ecosistemas marinos, fundamentalmente polares (agua y hielo de mar, sedimentos y lodos marinos, arena, etc.); ambientes de agua dulce (agua potable, agua y sedimentos de lagos y ríos, etc.); ecosistemas terrestres (suelo y rizosfera de plantas); entornos industriales (lodos activados, aguas residuales); ambientes alimentarios (productos lácteos, pescado, carne y productos de aves de corral); muestras clínicas humanas (esputo, sangre, saliva, orina, etc.); ambientes clínicos (equipos y dispositivos

hospitalarios); animales enfermos (perros, gatos, aves, peces de agua dulce y marinos, moluscos, equinodermos, crustáceos, etc.); algas; algunos insectos; entre otras.

3.1.3.1 Especies relacionadas con procesos clínicos

Aunque como se ha comentado anteriormente la mayoría de los miembros de la familia son microorganismos ambientales, existen algunas especies patógenas para el hombre y los animales (Tabla 3). Dentro de estas especies, aquellas que son capaces de infectar al hombre, aunque su prevalencia es bastante baja (se aíslan con una frecuencia del 1% o menor), tienen una importante significación clínica dado que suelen ser extremadamente resistentes a muchos agentes antimicrobianos (Jooste y Hugo, 1999), lo que limita enormemente las opciones terapéuticas.

Elizabethkingia meningoseptica es la especie de mayor relevancia clínica en el hombre. Es responsable de enfermedades invasivas en neonatos y niños e infecciones en adultos, fundamentalmente en pacientes inmunocomprometidos (Bernardet y col., 2006; Ceyhan y Celik, 2011; Isaac y Neetoo, 2011; Ghafur y col., 2013). También se ha aislado ocasionalmente de animales enfermos como gatos y perros, tortugas y serpientes, varias especies de aves, así como de diversas ranas y peces (Sims, 1974; Vancanneyt y col., 1994; Mauel y col., 2002; Miller y col., 2004; Bernardet y col., 2006).

Las especies *Empedobacter brevis*, *Weeksella virosa* y dos especies del género *Myroides* (*Myroides odoratus* y *Myroides odoratimimus*), son comunes en el entorno hospitalario como responsables de infecciones nosocomiales. Estos microrganismos son capaces de colonizar dispositivos permanentes, tales como catéteres, y posteriormente infectar a las personas causando diversas infecciones locales como celulitis o procesos septicémicos (Green y col., 2001; Hugo y col., 2006a, b; Maraki y col., 2012; Raman y col., 2012; Slenker y col., 2012). *E. brevis*, además, se ha relacionado con un caso de meningitis en un perro (Haburjak y Schubert, 1997).

Tabla 3. Principales microorganismos patógenos de la familia *Flavobacteriaceae*

Especies	Humanos	Perros y gatos	Aves	Peces	Reptiles y anfibios
Elizabethkingia meningoseptica	+	+	+	+	+
Empedobacter brevis	+	+			
Weeksella virosa	+				
Myroides odoratus *Myroides odoratimimus*	+				
Bergeyella zoohelcum	+	+			
Capnocytophaga spp.	+	+			
Coenonia anatina			+		
Riemerella spp.			+		
Ornithobacterium rhinotracheale			+		
Tenacibaculum maritimum *Tenacibaculum ovolyticum* *Tenacibaculum discolor* *Tenacibaculum soleae*				+	
Flavovacterium spp. *Flavovacterium psychrophilum* *Flavovacterium johnsoniae* *Flavovacterium branchiophilum* *Flavovacterium columnare*				+	
Chryseobacterium gleum *Chryseobacterium indologenes* *Chryseobacterium hominis*	+				
Chryseobacterium scophthalmum *Chryseobacterium joostei* *Chryseobacterium piscicola*				+	

Bergeyella zoohelcum y las especies del género *Capnocytophag*a están presente en la cavidad oral de los mamíferos y se aíslan de heridas humanas causadas por mordeduras de gatos y perros (Shukla y col., 2004; Hugo y col., 2006a). Además se asocian, aunque en menor medida, con casos de abscesos, meningitis y septicemia (Hugo y col., 2006a; Lin y col., 2007). Algunas especies del género *Capnocytophaga* también están implicadas en el hombre en enfermedades periodontales, gingivitis o caries dentales (Leadbetter, 2006; Jolivet-Gougeon y col., 2007; Piau y col., 2013). Asimismo*, B. zoohelcum* y *Capnocytophaga canimorsus*, se han asociado con infecciones respiratorias en gatos (Decostere y col., 2002; Lloret y col., 2013).

Las especies *Coenonia anatine*, *Ornithobacterium rhinotracheale* y dos especies del género *Riemerella* (*Riemerella anatipestifer* y *Riemerella columbina*) son responsables de neumonías y septicemias en diferentes especies de aves como patos y gansos, palomas, pavos y pollos (Vandamme y col., 1999; Szalay y col., 2002; Vandamme y col., 2006; Yu y col., 2008; Rubbenstroth y col., 2011).

Algunos miembros del género *Tenacibaculum* son reconocidos patógenos de peces. Así, *Tenacibaculum maritimum* es el agente causal de la tenacibaculosis, enfermedad que afecta a diferentes especies de peces marinos en todo el mundo (Avendaño-Herrera y col., 2006). De igual forma, *Tenacibaculum ovolyticum* es capaz de colonizar y ocasionar ulceras en la superficie de huevos, fundamentalmente en el halibut del atlántico en el que causó serias pérdidas en Noruega durante los años noventa (Hansen y col., 1992; Bergh y col., 2001; Míguez y Combarro, 2003). Otras especies de este género como *Tenacibaculum discolor* (Piñeiro-Vidal y col., 2008b) y *Tenacibaculum soleae* (Piñeiro-Vidal y col., 2008a), se han aislado de lenguados enfermos.

En los géneros *Flavobacterium* y *Chryseobacterium* existen también distintas especies patógenas a las que haremos referencia en los epígrafes 3.2.3.1 y 3.3.3.1, respectivamente.

3.1.4 Aplicaciones biotecnológicas

Varios de los microorganismos que conforman la familia *Flavobacteriaceae* podrían tener importantes aplicaciones biotecnológicas en el futuro. Por ejemplo podrían ser utilizados en la industria alimentaria y farmacéutica por la capacidad que tienen algunos para degradar moléculas complejas como la queratina o la

quitina, o complejos polisacáridos de la pared celular de las algas marinas, como el agar y los carragenanos (Cottrell y Kirchman, 2000; Barbeyron y col., 2001; Riffel y Brandelli, 2002; Riffel y col., 2003; Cottrell y col., 2005; Gomez-Pereira y col., 2012; Gurav y Jadhav, 2013; Mann y col., 2013). Del mismo modo, otros miembros de la familia son capaces de degradar productos químicos tóxicos, tales como arsénico, anilina, furano, perclorato, atrazina, pentaclorofenol, oligómeros de nylon, pesticidas e hidrocarburos aromáticos policíclicos, por lo que podrían tener importantes aplicaciones en la biorremediación (McAllister y col., 1996; Lee y Xun, 1997; Lo y col., 1998; Negoro, 2000; Wang y Vipulanandan, 2001; Bodour y col., 2004; Zhou y col., 2007; Cheng y col., 2010; Sun y col., 2011).

En la familia también existen algunas especies psicrófilas, aisladas de ambientes fríos o polares, que producen enzimas potencialmente interesantes tales como proteasas *cold-active* o endopeptidasas que podrían tener múltiples aplicaciones en diversos sectores industriales y biotecnológicos como en el procesamiento industrial de alimentos, cuero y de productos farmacéuticos (Chen y col., 2013a; Zhou y col., 2013). Del mismo modo, se han descrito otros miembros de la familia que presentan capacidad algicida por lo que podrían tener un papel beneficioso por ejemplo en la destrucción de dinoflagelados tóxicos (Chen y col., 2012b; Thomas y col., 2012, 2013; Mann y col., 2013).

3.2 Género *Flavobacterium*

3.2.1 Aspectos taxonómicos

El género *Flavobacterium* se estableció en 1923 para delimitar un grupo heterogéneo de microorganismos recuperados de diferentes entornos que se caracterizaban por ser mayoritariamente bacilos Gram negativos (también se incluyeron algunas especies Gram positivas), aerobios, quimiorganótrofos, inmóviles o móviles por flagelos polares o flagelos peritricos, no formadores de endosporas y que crecían en medios de cultivo dando lugar a colonias pigmentadas de color amarillo. El género quedó constituido inicialmente por 46 especies (Bergey y col., 1923).

En sucesivas ediciones del *Bergey's Manual of Determinative Bacteriology* la heterogeneidad del género se redujo gradualmente. De esta forma, en la quinta edición, se excluyeron del género los microorganismos con flagelos polares (Bergey y col., 1939). En la sexta edición (Bergey y Breed, 1948) el género fue

ubicado en la familia *Achromobacteriaceae* y en la séptima (Weeks y Breed, 1957) se eliminaron las especies Gram positivas. Posteriormente, en la octava edición (Weeks, 1974) el género se dividió en dos secciones: la sección I que agrupaba las especies con un contenido G+C del ADN de 30-42 mol% (especies con bajo contenido G+C) y la sección II que incluía especies con un contenido G+C del ADN de 63-70 mol% (especies con alto contenido G+C).

Con posterioridad, McMeekin y Shewan (1978) propusieron que el género *Flavobacterium* sólo debería incluir los microorganismos desprovistos de *gliding*, no móviles y con bajo porcentaje de G+C del ADN. En este mismo sentido, Holmes y Owen (1979) consideraron que la descripción del género dada por Weeks (1974) debía ser corregida. Así, basándose en los resultados de varios estudios que incluían aislamientos de origen clínico (Lapage y Owen, 1973; Owen y Snell, 1973; Owen y Lapage, 1974; Owen y Snell, 1976; Holmes y col., 1977, 1978, 1979) y no clínico (Hayes, 1977; Hayes y col., 1977), propusieron una modificación del género con el fin de limitarlo a aquellos microorganismos clínicos, ambientales o de alimentos, que fuesen Gram negativos, aerobios, quimiorganótrofos, oxidasa y catalasa positivos, no móviles, que desarrollaban colonias generalmente pigmentadas de color amarillo-naranja y con un tipo de metabolismo estrictamente respiratorio, activamente proteolíticos pero pobremente sacarolíticos y con un contenido G+C del ADN de 31-40 mol%. Esta propuesta quedó recogida en la primera edición del *Bergey's Manual of Systematic Bacteriology* (Holmes y col., 1984a), quedando constituido el género por siete especies (*Flavobacterium aquatile*, [*Flavobacterium*] *balustinum*, [*Flavobacterium*] *breve*, [*Flavobacterium*] *meningosepticum*, [*Flavobacterium*] *multivorum*, [*F.*] *odoratum*, y [*Flavobacterium*] *spiritivorum*).

Un poco más tarde, en la segunda edición de *The Prokaryotes*, Holmes (1992) dividió las especies del género en cuatro grupos (A, B, C, D), en base a sus características fenotípicas excluyendo a la especie tipo, *F. aquatile*, por ser atípica en comparación con el resto de especies incluidas en el género. En 1979, Holmes y Owen habían propuesto que *F. aquatile* fuese rechazada y sustituida por [*F*]. *breve* como la especie tipo, solicitud que fue denegada por La Comisión Judicial del Comité Internacional de Bacteriología Sistemática (Wayne, 1982).

Según la clasificación de Holmes (1992), el grupo A estaba formado por [*F*]. *balustinum*, [*F*]. *breve*, [*Flavobacterium*] *gleum*, [*Flavobacterium*] *indologenes*,

[*Flavobacterium*] *indoltheticum*, y [*F*]. *meningosepticum*, que fueron transferidos posteriormente a los nuevos géneros *Chryseobacterium* y *Empedobacter* (Vandamme y col., 1994a). El grupo B lo componía [*F*]. *odoratum*. El grupo C incluía a [*Flavobacterium*] *mizutae*, [*F*]. *multivorum*, [*F*]. *spiritivorum*, [*Flavobacterium*] *thalpophilum*, y [*Flavobacterium*] *yabuuchiae*; estos microorganismos formaron un grupo considerado como un género separado para el cual se propuso el nombre *Sphingobacterium* (Yabuuchi y col., 1983; Takeuchi y Yokota, 1992). El grupo D lo constituían dos especies inicialmente conocidas como grupo CDC IIf y IIj, para las cuales se propuso la creación del género *Weeksella* (Holmes y col., 1986), y pasaron a nombrarse *W. virosa* y [*Weeksella*] *zoohelcum* (ahora *B. zoohelcum*; Vandamme y col., 1994a) respectivamente. Posteriormente fueron descritos nuevos microrganismos pertenecientes al género *Flavobacterium* (Wakabayashi y col., 1989; Dobson y col., 1993), estando constituido en el año 1996, por seis especies válidas: *F. aquatile* (la especie tipo), *Flavobacterium branchiophilum*, [*Flavobacterium*] *ferrugineum*, [*F*.] *gondwanense*, [*F*]. *odoratum*, y [*F*.] *salegens*.

En este año, 1996, se publicó un extenso estudio polifásico que abarcó 106 cepas bacterianas pertenecientes principalmente a los géneros *Cytophaga*, *Flexibacter*, *Flavobacterium*, *Sphingobacterium* y *Microscilla* (Bernardet y col., 1996). En esta investigación, estudios de hibridación ADN-ARNr permitieron establecer una clara delimitación de un grupo de microorganismos mal clasificados relacionados con *F. aquatile*, denominado "*Flavobacterium aquatile* rRNA cluster", que contenía las especies *F. aquatile*, *F. branchiophilum*, [*Cytophaga*] *flevensis*, [*Cytophaga*] *aquatilis*, [*Cytophaga*] *johnsonae*, [*Cytophaga*] *pectinovora*, [*Cytophaga*] *saccharophila*, [*Cytophaga*] *succinicans* "*Cytophaga allerginae*", "*Cytophaga xantha*", [*Flexibacter*] *columnaris*, [*Flexibacter*] *psychrophilus*, [*F*] *aurantiacus*, "*F. aurantiacus* subsp. *excathedrus*", "*Promyxobacterium flavum*", "*Sporocytophaga cauliformis*" y [*F*]. *odoratum*. Las especies [*F*]. *ferrugineum*, [*F*]. *gondwanens* y [*F*]. *salegens*, que hasta ese momento formaban parte del género *Flavobacterium*, quedaron claramente separadas del "*Flavobacterium aquatile* rRNA cluster", por lo que se propuso que fuesen excluidas del género, estando ubicadas actualmente en los géneros *Terrimonas* (Xie y Yokota, 2006), *Psychroflexus* (Bowman y col., 1998) y *Salegentibacter* (McCammon y Bowman, 2000), respectivamente.

Todas las especies agrupadas en el "*Flavobacterium aquatile* rRNA cluster" además de tener hábitats similares (suelo y agua dulce), también compartían

características como la presencia de MK-6 como principal quinona respiratoria, perfiles de ácidos grasos muy similares, un contenido G+C del ADN entre 32 y 37 mol%, además de varias características fenotípicas comunes a la mayoría de las especies como por ejemplo la presencia de *gliding motility*, la producción de pigmento de color amarillo de los tipos carotenoide o flexirrubina o la producción de ácido a partir de carbohidratos. Estos criterios, fenotípicos y filogenéticos, apoyaron la inclusión de la mayoría de estos microorganismos en el género *Flavobacterium* con *F. aquatile* como especie tipo (Bernardet y col., 1996). La única excepción fue [*F*]. *odoratum*, que había sido incluido en el *cluster* debido a la baja resolución de la secuenciación del gen 16S ARNr utilizada en ese momento, pero que difería de las otras especies del *cluster* en varias características y en su origen clínico, este microorganismo, un poco más tarde fue reclasificado en un género separado, *Myroides*, como *M. odoratus* (Vancanneyt y col., 1996).

En este estudio (Bernardet y col., 1996), también se corrigieron algunos problemas de nomenclatura relacionados con los nombres de algunos de los microorganismos reclasificados dentro del género para los cuales se propusieron nuevas combinaciones. Así, [*C.*] *aquatilis* fue reclasificada como *Flavobacterium hydatis* para evitar que la nueva combinación se convirtiese en un homónimo de *F. aquatile*; la reclasificación de [*C.*] *johnsonae* fue corregido por su epíteto específico *johnsoniae*; las cepas de [*F.*] *aurantiacus* compartían muchas características fenotípicas, similares perfiles de ácidos grasos, y altos niveles de la relación ADN-ADN con la cepa tipo de *Flavobacterium johnsoniae*, por lo que se propuso que fueran incluidas en esta especie. Finalmente, se decidió referir a algunos taxones, tales como "*Cytophaga allerginae*", "*Cytophaga xantha*", "*F. aurantiacus* subsp. *excathedrus*", "*Promyxobacterium flavum*", y "*Sporocytophaga cauliformis*", que claramente pertenecían al género *Flavobacterium* pero que no habían sido válidamente publicados, como *Flavobacterium* sp. "*Cytophaga xantha*", fue más adelante válidamente publicada como *Flavobacterium xanthum* como resultado de un amplio estudio realizado con aislados de la Antártida (McCammon y Bowman, 2000).

Como consecuencia de este estudio (Bernardet y col., 1996), el género quedó formado por diez especies: *F. aquatile*, *F. branchiophilum*, *Flavobacterium columnare*, *Flavobacterium flevense*, *F. hydatis*, *F. johnsoniae*, *Flavobacterium pectinovorum*, *F. psychrophilum*, *Flavobacterium saccharophilum* y *Flavobacterium succinicans*.

Desde entonces, el género *Flavobacterium* se ha expandido considerablemente debido a la descripción de muchas nuevas especies (Figura 2), estando formado en el momento de escribir esta memoria, octubre de 2014, por 107 especies válidamente publicadas, cuyos nombres se relacionan en la Tabla 4 (las especies que se describen en este estudio no están incluidas).

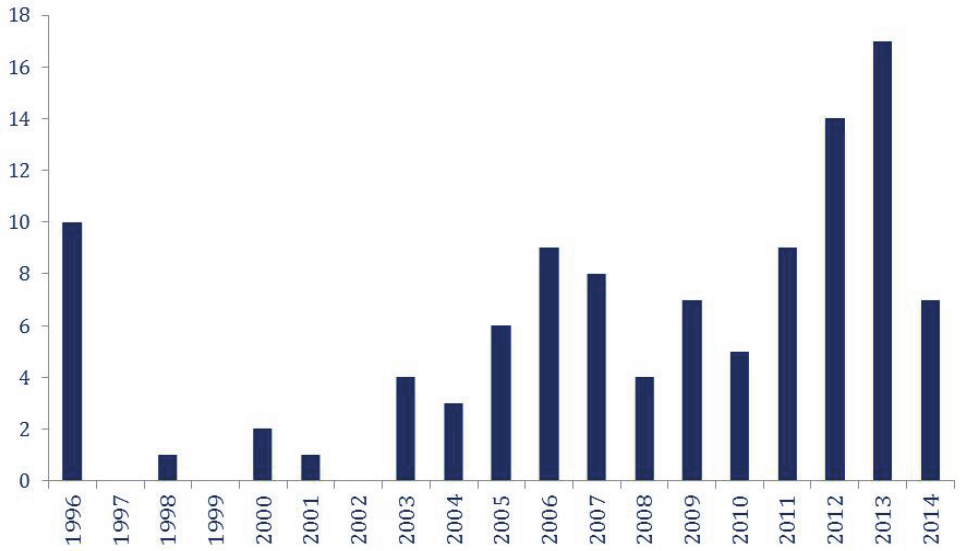

Figura 2. Número de especies válidamente publicadas en el género *Flavobacterium* desde el año 1996 hasta octubre de 2014.

Existen en el género algunas especies que según la secuenciación del gen 16S ARNr no pertenecen al mismo y cuya taxonomía aún no se ha corregido oficialmente. Tal es el caso de las especies *Flavobacterium devorans* (99,86% de similitud con *Sphingomonas paucimobilis*); *Flavobacterium acidificum* (99,9% de similitud con *Pantoea ananatis*); *Flavobacterium oceasnosedimentum* (99,8% de similitud con *Curtobacterium citreum*); y *Flavobacterium thermophilum* (99,7% de similitud con *Anoxybacillus kamchatkensis*). Por otra parte, la especie *Flavobacterium sasangense* (Yoon y col., 2009) fue invalidada un año más tarde de su descripción ya que no se presentaron pruebas de haber depositado la cepa tipo en dos colecciones de cultivos en diferentes países, como se requiere para la publicación válida del nombre de una especie según el Código Bacteriológico (Euzeby y Parte, 2014c).

Tabla 4. Especies validadas del Género *Flavobacterium* indicando su origen y lugar de aislamiento

Especie	Fuente	Referencia
Flavobacterium aciduliphilum	Agua dulce de lago artificial. Corea.	Kang y col., 2013
Flavobacterium akiainvivens	Madera en descomposición. Hawái.	Kuo y col., 2013
Flavobacterium algicola	Algas marinas. Japón.	Miyashita y col., 2010
Flavobacterium anatoliense	Agua dulce. Turquía.	Kacagan y col., 2013
Flavobacterium anhuiense	Suelo. China.	Liu y col., 2008
Flavobacterium antarcticum	Suelo. Antártida.	Yi y col., 2005a
Flavobacterium aquaticum	Agua de un campo de arroz. India.	Subhash y col., 2013
Flavobacterium aquatile	Agua de pozo. Inglaterra.	Sheu y col., 2013.
Flavobacterium aquidurense	Agua de manantial. Alemania.	Cousin y col., 2007
Flavobacterium araucananum	Salmones del Atlántico. Chile.	Kämpfer y col., 2012
Flavobacterium banpakuense	Compost de hojas y ramas. Japón.	Kim y col., 2011
Flavobacterium beibuense	Sedimentos marinos. China.	Dong y col., 2013b
Flavobacterium branchiophilum	Branquias de alevines enfermos de Yamame. Japón.	Bernardet y col., 1996
Flavobacterium caeni	Lodos activados de birreactor. China.	Fujii y col., 2014
Flavobacterium cauense	Sedimentos del lago Taihu. China.	Sheu y col., 2013
Flavobacterium ceti	Pulmones e hígado de ballenas picudas. España.	Kacagan y col., 2013.
Flavobacterium cheniae	Sedimentos de embalse. China.	Joung y col., 2013
Flavobacterium cheonanense	Depósito de agua dulce. Corea.	Lee y col., 2012b.
Flavobacterium cheonhonense	Depósito de agua dulce. Corea.	Lee y col., 2012a

Tabla 4. Continuación

Especie	Fuente	Referencia
Flavobacterium chilense	Trucha arcoíris. Chile.	Kämpfer y col., 2012
Flavobacterium chungangense	Lago de agua dulce. Corea.	Kim y col., 2009
Flavobacterium chungbukense	Suelo. Corea.	Lim y col., 2011
Flavobacterium chungnamense	Depósito de agua dulce. Corea.	Lee y col., 2012b.
Flavobacterium columnare	Salmón Chinook enfermo. EE.UU.	Bernardet y col., 1996
Flavobacterium compostarboris	Hojas y ramas de compost. Japón.	Kim y col., 2012
Flavobacterium croceum	Lodos activados. Corea.	Park y col., 2006
Flavobacterium cucumis	Suelo de invernadero. Corea.	Weon y col., 2007
Flavobacterium cutihirudinis	Piel de sanguijuela. Alemania.	Glaeser y col., 2013
Flavobacterium daejeonense	Suelo de invernadero. Corea.	Kim y col., 2006a
Flavobacterium dankookense	Depósito de agua dulce. Corea.	Lee y col., 2012b
Flavobacterium defluvii	Lodo activado, planta de tratamiento de aguas residuales. Corea.	Chen y col., 2013b
Flavobacterium degerlachei	Tapetes microbianos. Antártida.	Van Trappen y col., 2004
Flavobacterium denitrificans	Intestino de lombriz de tierra. Alemania.	Horn y col., 2005
Flavobacterium dongtanense	Rizosfera. China.	Joung y col., 2013
Flavobacterium enshiense	Suelo, planta de tratamiento de residuos líquidos. China.	Dong y col., 2013a
Flavobacterium faecale	Heces de pingüinos. Antártida	Kim y col., 2014

Tabla 4. Continuación

Especie	Fuente	Referencia
Flavobacterium filum	Planta de tratamiento de aguas residuales. Corea.	Liy col., 2014
Flavobacterium flevense	Agua dulce. Países Bajos.	Bernardet y col., 1996
Flavobacterium fluvii	Sedimentos de arroyo. Corea.	Lee y col., 2010
Flavobacterium fontis	Estanque de agua dulce. Corea.	Chun y col., 2013
Flavobacterium frigidarium	Sedimentos marinos. Antártida.	Humphry y col., 2001
Flavobacterium frigidimaris	Agua de mar. Antártida.	Nogi y col., 2005
Flavobacterium frigoris	Tapetes microbianos. Antártida.	Van Trappen y col., 2004
Flavobacterium fryxellicola	Tapetes microbianos. Antártida.	Van Trappen y col., 2005
Flavobacterium gelidilacus	Tapetes microbianos. Antártida.	Joung y col., 2013
Flavobacterium gillisiae	Hielo marino. Antártida.	McCammon y Bowman, 2000
Flavobacterium ginsengisoli	Suelo de campo de ginseng. Corea.	Kim y col., 2013
Flavobacterium ginsenosidimutans	Suelo de campo de ginseng. Corea.	Yang y col., 2011
Flavobacterium glaciei	Glaciar. China.	Zhang y col., 2006
Flavobacterium glycines	Rizosfera de cultivo de soja. India.	Madhaiyan y col., 2010
Flavobacterium granuli	Gránulos utilizados en planta de tratamiento de aguas residuales. Corea.	Aslam y col., 2005
Flavobacterium haoranii	Lodos activados, tratamiento de aguas residuales. China.	Sheu y col., 2013
Flavobacterium hauense	Suelo. China.	Dong y col., 2013b

Tabla 4. Continuación

Especie	Fuente	Referencia
Flavobacterium hercynium	Agua de manantial. Alemania	Kim y col., 2012
Flavobacterium hibernum	Lago. Antártida.	McCammon y col., 1998
Flavobacterium hydatis	Salmón enfermo. EE.UU.	Bernardet y col., 1996
Flavobacterium indicum	Agua de fuente termal. India.	Saha y Chakrabarti, 2006
Flavobacterium johnsoniae	Suelo. Inglaterra.	Chen y col., 2013b
Flavobacterium jumunjinense	Agua de laguna. Corea.	Joung y col., 2013
Flavobacterium koreense	Depósito de agua dulce. Corea.	Lee y col., 2012b
Flavobacterium kyungheense	Suelo de campo de ginseng. República de Corea.	Son y col., 2013
Flavobacterium lacus	Agua de lago salado. China.	Li y col., 2014
Flavobacterium limicola	Sedimentos de agua dulce. Japón.	Tamaki y col., 2003
Flavobacterium limnosediminis	Sedimentos de lago de agua dulce. República de Corea.	Lee y col., 2013
Flavobacterium lindanitolerans	Suelo. India.	Jit y col., 2008
Flavobacterium longum	Lodos, planta de tratamiento de aguas residuales.	Fujii y col., 2014
Flavobacterium macrobrachii	Agua dulce. Taiwán.	Sheu y col., 2011
Flavobacterium marinum	Agua de mar. Océano Índico.	Song y col., 2013
Flavobacterium micromati	Tapetes microbianos. Antártida.	Van Trappen y col., 2004
Flavobacterium myungsuense	Agua de lago de agua dulce. Corea.	Joung y col., 2012
Flavobacterium nitratireducens	Agua marina. India.	Nupur y col., 2013

Tabla 4. Continuación

Especie	Fuente	Referencia
Flavobacterium noncentrifugens	Glaciar. China.	Zhu y col., 2013
Flavobacterium omnivorum	Glaciar. China.	Zhu y col., 2003
Flavobacterium pectinovorum	Suelo. Inglaterra.	Bernardet y col., 1996
Flavobacterium phragmitis	Raíces de cañas. China.	Liu y col., 2011
Flavobacterium ponti	Agua de mar. Corea.	Yoon y col., 2011
Flavobacterium psychrolimnae	Tapetes microbianos. Antártida.	Van Trappen y col., 2005
Flavobacterium psychrophilum	Salmón coho. EEUU.	Bernardet y col., 1996
Flavobacterium rakeshii	Sedimento marino. Mar de Arabia.	Kaur y col., 2012
Flavobacterium reichenbachii	Rio de agua dura. Alemania.	Ali y col., 2009
Flavobacterium resistens	Sedimentos de arroyo. Corea	Kim y col., 2012
Flavobacterium rivuli	Rio de agua dura. Alemania.	Dong y col., 2013b
Flavobacterium saccharophilum	Río. Inglaterra.	Bernardet y col., 1996
Flavobacterium saliperosum	Sedimentos de lago de agua dulce. China.	Dong y col., 2013a
Flavobacterium segetis	Suelo. Antártida.	Yi y Chun, 2006
Flavobacterium sinopsychrotolerans	Glaciar. China.	Xu y col., 2011
Flavobacterium soli	Suelo. Corea.	Yoon y col., 2006
Flavobacterium spartansii	Riñones de Salmón Chinook. EEUU.	Loch y Faisal, 2014b
Flavobacterium squillarum	Estanque de cultivo de camarón (agua dulce). Taiwán.	Sheu y col., 2013
Flavobacterium subsaxonicum	Rio de agua dura. Alemania.	Dong y col., 2013b

Tabla 4. Continuación

Especie	Fuente	Referencia
Flavobacterium succinicans	Aleta caudal alevines de Salmón chinook. EEUU.	Bernardet y col., 1996
Flavobacterium suncheonense	Suelo de invernadero. Corea.	Dong y col., 2013a.
Flavobacterium swingsii	Rio de agua dura. Alemania.	Ali y col., 2009
Flavobacterium tegetincola	Tapetes de cianobacterias, lago de salinidad marina. Antártida.	McCammon y Bowman, 2000
Flavobacterium terrae	Suelo de invernadero. Corea.	Sheu y col., 2013
Flavobacterium terrigena	Suelo. Corea.	Fujii y col., 2014
Flavobacterium tiangeerense	Glaciar. China.	Xin y col., 2009
Flavobacterium tilapiae	Estanque de cultivo de tilapia (agua dulce). Taiwán	Chen y col., 2013b
Flavobacterium ummariense	Suelo. India.	Lata y col., 2012
Flavobacterium urocaniciphilum	Lodos, planta de tratamiento de aguas residuales.	Fujii y col., 2014
Flavobacterium urumqiense	Glaciar. China.	Dong y col., 2012
Flavobacterium weaverense	Suelo. Antártida.	Yi y Chun, 2006
Flavobacterium xanthum	Barro. Antártida.	McCammon y Bowman, 2000
Flavobacterium xinjiangense	Glaciar. China.	Zhu y col., 2003
Flavobacterium xueshanense	Glaciar. China.	Dong y col., 2012
Flavobacterium yanchengense	Suelo. China.	Hu y col., 2013
Flavobacterium yonginense	Agua de lago artificial. Corea.	Joung y col., 2012

La información detallada, incluyendo sinónimos, datos históricos de los nombres, cepas tipo, etc., puede obtenerse a través de la *List of Prokaryotic names with Standing in Nomenclature*, disponible en la dirección http://www.bacterio.net/flavobacterium.html (Euzeby y Parte, 2014c). En aquellos nombres derivados de nuevas combinaciones o cuyas descripciones han sido corregidas solo se muestra la referencia más reciente.

3.2.2 Características fenotípicas, genotípicas y quimiotaxonómicas

Las características fenotípicas comunes a todos los miembros del género *Flavobacterium* son pocas, esto se debe a que el género contiene, con diferencia, el mayor número de especies de la familia *Flavobacteriaceae*, por lo que muchas de las características fenotípicas que se estudian para diferenciar los género de la familia, también pueden variar dentro del género *Flavobacterium* y por lo tanto, no se pueden utilizar para delimitarlo de otros géneros de la familia.

Según la descripción del género (Bernardet y col., 1996; Bernardet y Bowman, 2006), las especies que lo forman son bacilos individuales con lados paralelos o ligeramente curvados y extremos redondeados o ligeramente cónicos. Una excepción es la especie *Flavobacterium denitrificans* que puede formar cadenas de 3-14 células (Horn y col., 2005). Por lo general, los microorganismos agrupados en el género tienen un diámetro aproximado de 0,3 a 0,5 µm, y una longitud variable, a menudo de 2 a 5 µm, aunque bajo ciertas condiciones de crecimiento es posible observar células más pequeñas (1 µm) o filamentosas y flexibles (10 a 40 µm). Son bacterias Gram negativas, que no forman endosporas, y no presentan flagelos, aunque varias especies del género son móviles por *gliding*.

Estos microorganismos crecen fácilmente en medios comerciales tales como agar nutritivo, agar tripticasa soja y los medios de cultivo líquidos correspondientes. Sin embargo algunas especies como por ejemplo las especies patógenas de peces (*F. psychrophilum*, *F. branchiophilum* y *F. columnare*) necesitan para su crecimiento medios con bajas concentraciones de nutrientes, tales como *Anacker and Ordal* (Anacker y Ordal, 1959), R2A (Reasoner y Geldreich, 1985) o el medio *Shieh* modificado (Song y col., 1988).

En medios sólidos ricos en nutrientes, las especies del género forman colonias circulares, de moderadamente convexas a convexas, de translúcidas a opacas, lisas y brillantes, con bordes enteros u ondulados. En medios sólidos pobres en nutrientes crecen dando lugar a colonias que pueden ser planas y presentar expansión en la superficie del agar (*spreading*), con márgenes irregulares, rizoides o filamentosos. Generalmente poseen un color amarillo brillante o naranja debido a la producción de pigmentos de tipo carotenoide o flexirrubina o ambos. Algunas especies del género también producen un pigmento difusible de color rosa a marrón oscuro cuando se cultivan en agar tirosina, tal es el

caso de *Flavobacterium anatoliense* (Kacagan y col., 2013), *Flavobacterium aquidurense* (Cousin y col., 2007) y *Flavobacterium caeni* (Liu y col., 2010).

Teniendo en cuenta los requerimientos de cloruro sódico (NaCl), casi todas las especies del género crecen en medios que contengan entre un 2-4% de NaCl. La tolerancia más alta al NaCl (9%) la presentan las especies *Flavobacterium frigidarium* (Humphry y col., 2001) y *Flavobacterium beibuense* (Fu y col., 2011). Por el contrario, otras especies recuperadas de suelo y agua dulce, incluyendo las especies patógenas de peces, son bastantes sensibles al NaCl y no crecen en agar marino (1,9% de NaCl). Así, *F. columnare* tolera hasta 0,5% de NaCl (Bernardet y Grimont, 1989) y *F. branchiophilum* y *F. psychrophilum* hasta 0,2 y 0,5-1%, respectivamente (Bernardet y Kerouault, 1989; Holt y col., 1989; Ostland y col., 1994). En otras especies, como por ejemplo *Flavobacterium croceum*, *Flavobacterium limicola* y *Flavobacterium saliperosum*, el crecimiento se ve inhibido por concentraciones de NaCl del 1-1,5% (Tamaki y col., 2003; Park y col., 2006a; Dong y col., 2013a).

La temperatura óptima de crecimiento de estos microorganismos puede variar en función de la especie bacteriana. Así, los microorganismos aislados de ambientes fríos o polares, la mayoría psicrotolerantes y no verdaderos organismos psicrófilos, tienen un rango de temperatura óptima de crecimiento que varía entre 11-22 °C. Una excepción es la especie *Flavobacterium hibernum*, que aunque se aisló de la Antártida, su temperatura óptima de crecimiento es 26 °C y también crece a la temperatura más baja notificada dentro del género, -7 °C (McCammon y col., 1998). Por otro parte, las especies aisladas de ambientes templados crecen óptimamente en un rango de temperatura de 10-25 °C, aunque algunas son capaces de crecer a temperaturas tan bajas como 0 °C o tan altas como 45 °C (Park y col., 2006a). La única especie del género recobrada de un ambiente cálido (agua de manantial a 37-38 °C), *Flavobacterium indicum*, presenta una temperatura óptima de crecimiento de 37 °C (Saha y Chakrabarti, 2006).

De forma general, la mayoría de las especies del género son quimiorganótrofas, estrictamente aerobias con metabolismo de tipo respiratorio con el oxígeno como aceptor final de electrones (Bernardet y col., 1996; Bernardet y Bowman, 2006, 2011). Sin embargo, algunas especies como *F. hydatis*, *F. succinicans, F. denitrificans, Flavobacterium lindanitolerans* y *Flavobacterium ponti* pueden crecer en condiciones de anaerobiosis (Stanier, 1947; Anderson y Ordal,

1961; Chase, 1965; Strohl y Tait, 1978; Reichenbach, 1989; Horn y col., 2005; Bernardet y Bowman, 2006; Yoon y col., 2011). De igual forma, las especies *Flavobacterium ceti*, *F. croceum*, *Flavobacterium defluvii*, *Flavobacterium filum*, *Flavobacterium segetis* y *Flavobacterium weaverense* son capaces de crecer en condiciones de anaerobiosis aunque de forma débil y retardada (Park y col., 2006a; Yi y Chun, 2006; Park y col., 2007; Ryu y col., 2007; Vela y col., 2007).

Todas las especies del género producen catalasa, la mayoría citocromo oxidasa y sólo unas pocas presentan actividad β-galactosidasa. La producción de indol es negativa en todas las especies en las que ha sido estudiada. La mayoría de las especies no producen ácido sulfídrico, excepto algunas especies como por ejemplo *Flavobacterium cauense*, *Flavobacterium cheniae*, *F. columnare*, *Flavobacterium reichenbachii*, *Flavobacterium rivuli*, *F. saccharophilum*, *Flavobacterium subsaxonicum*, *Flavobacterium suncheonense*, *F. xanthum* y *Flavobacterium xinjiangense*. Casi todas las especies degradan gelatina y caseína y varias especies también hidrolizan almidón y esculina. Otros polisacáridos, tales como quitina, pectina y carboximetilcelulosa, sólo son hidrolizados por unas pocas especies y las únicas especies agarolíticas son *F. flevense*, *F. limicola*, y *F. saccharophilum*. No descomponen la celulosa cristalina (papel de filtro) (Bernardet y Bowman, 2006). La capacidad sacarolítica varía ampliamente entre los miembros del género. Algunas especies son capaces de utilizar una amplia gama de hidratos de carbono (por ejemplo, *F. flevense*, *F. frigidimaris*, *F. hydatis*, *F. johnsoniae*, *F. pectinovorum* y *F. saccharophilum*), mientras que otros utilizan pocos o ningún carbohidrato (por ejemplo, *Flavobacterium antarcticum*, *F. branchiophilum, F. columnare, Flavobacterium gelidilacus*, *Flavobacterium glaciei*, *F. indicum*, *Flavobacterium micromati*, *F. psychrophilum, F. saliperosum*, *F. suncheonense*, *Flavobacterium tegetincola*, *F. weaverense*). Alrededor de la mitad de las especies de *Flavobacterium* degradan Tween y tirosina, mientras que sólo unas pocas especies son capaces de degradar el ADN y la urea. El contenido de G+C del ADN de las especies englobadas en el género es de 30-41 mol%, similar al de la mayoría de los otros miembros de la familia *Flavobacteriaceae*, aunque las especie *F. subsaxonicum* (Ali y col., 2009) y *F. caeni* (Liu y col., 2010) presenta valores más altos de 43,3 y 52±0,6 mol%, respectivamente.

Existen una serie de características fenotípicas recomendadas para la descripción de nuevas especies del género publicadas por Bernardet y col. (2002). Estas incluyen: morfología de las colonias en agar *Anacker and Ordal*, adherencia

de las colonias al agar, adsorción del rojo Congo de las colonias, crecimiento en agar marino, agar nutritivo y agar tripticasa soja, crecimiento a 25 °C, presencia de *gliding motility*, producción de pigmento tipo flexirrubina, utilización de la glucosa como única fuente de carbono y energía, producción de ácido a partir de carbohidratos aeróbicamente, degradación de esculina, agar, alginato, carboximetilcelulosa, caseína, quitina, ADN, gelatina, pectina, almidón, L-tirosina, y urea, producción de pigmento difusible marrón en agar L-tirosina, formación de precipitado en agar yema de huevo, actividad β-galactosidasa, susceptibilidad al O129, producción de H_2S, producción de citocromo oxidasa y reducción de nitratos.

En cuanto a las características quimiotaxonómicas, como en el resto de miembros de la familia *Flavobacteriaceae*, la menaquinona MK-6 es la única o predominante quinona respiratoria, no poseen esfingofosfolípidos y la homoespermidina es la principal poliamina presente en estos microorganismos (Bernardet y Bowman, 2011). Recientemente se ha hecho una corrección en el género indicando que el principal lípido polar es la fosfatidiletanolamina (Dong y col., 2013).

Con respecto al perfil de ácidos grasos, actualmente es imposible evaluar si todas las especies muestran perfiles de ácidos grasos generalmente similares o si existe un perfil específico de especie. Esto se debe principalmente a la heterogeneidad en las condiciones de cultivo utilizadas para el análisis de ácidos grasos, ya que tanto el medio utilizado para el crecimiento como la temperatura y el tiempo de incubación influyen en la composición de los ácidos grasos. No obstante, según Kang y col. (2013) en el género *Flavobacterium* los ácidos grasos predominantes son iso-$C_{15:0}$, iso-$C_{15:1}$ G, iso-$C_{15:0}$ 3-OH, iso-$C_{16:0}$ 3-OH e iso-$C_{17:0}$ 3-OH.

3.2.3 Ecología

Los miembros del género *Flavobacterium* habitan una gran variedad de ecosistemas de suelo y agua dulce, hábitats marinos o salinos, ambientes cálidos, templados o polares (Bernardet y Bowman, 2006).

De las 107 especies válidamente publicadas que conforman el género (octubre 2014; Euzeby y Parte, 2014c), la mayoría, 32 especies, se han originado en ambientes de agua dulce y 21 se han aislado de diferentes ecosistemas de suelo.

En menor medida se han descrito especies de entornos marinos (agua de mar, hielo marino, sedimentos marinos), agua de lago salino, peces y glaciares. Una única especie, *F. indicum*, ha sido aislada de un hábitat cálido (agua de fuente termal a 37-38 °C). Así, dieciocho especies han sido recuperadas de hábitats polares o fríos, y ocho de ambientes con salinidad moderada, aunque, hasta el momento, no se ha descrito ninguna especie del género aislada de ambientes hipersalinos. En la Tabla 4 se especifican todos los hábitats de los que se aislaron las cepas tipo de las especies del género y en la Figura 3 se muestra el número de especies descritas según la fuente de aislamiento.

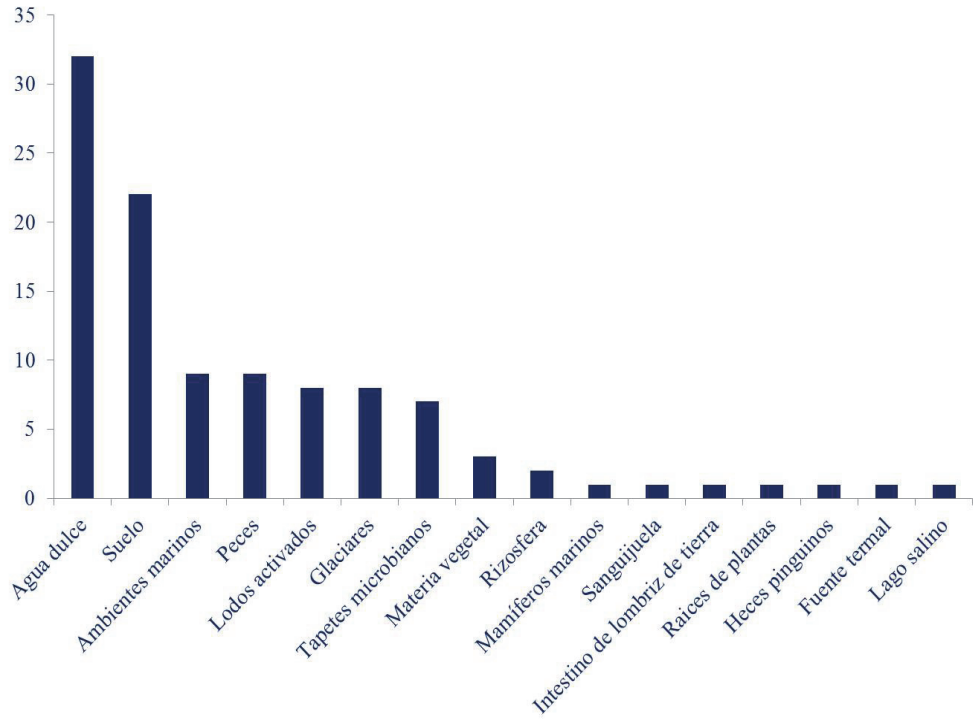

Figura 3. Número de especies del género *Flavobacterium* según la fuente de aislamiento.

Microorganismos pertenecientes al género *Flavobacterium* se han encontrado también en varios estudios de comunidades bacterianas en diferentes entornos, tal es el caso de una investigación realizada en Egipto sobre la diversidad bacteriana en diferentes ambientes salinos encontrando que parte de la comunidad bacteriana en estas muestras estaba formada por microorganismos del genero *Flavobacterium* (Ghozlan y col., 2006). De la misma forma, se han encontrado cepas de *Flavobacterium* spp. entre las bacterias que colonizan la

superficie y el interior de las raíces de orquídeas (Tsavkelova y col., 2007), y en un estudio sobre la comunidad bacteriana asociada a las raíces de plantas de tomate se encontró que el 87% de las bacterias también pertenecía a miembros no descritos del género *Flavobacterium* (Kim y col., 2006b). Además, algunos aislados recuperados de permafrost en Siberia y China también han sido identificados como *Flavobacterium* spp. (Bai y col., 2006; Vishnivetskaya y col., 2006).

Otros hábitats en los que se han identificado microorganismos del género *Flavobacterium* son: el bacterioplacton de lagos (Gich y col., 2005; Eiler y Bertilsson, 2007); el intestino de algunas larvas de mariposas (Xiang y col., 2006); larvas de escarabajos (Bahar y Demirbag, 2007); en amebas (Müller y col., 1999; Horn y col., 2001) y en el agua y los sedimentos de los sistemas de cultivo intensivo de peces (Bernardet y Bowman, 2006).

3.2.3.1 Especies relacionadas con procesos clínicos en el hombre y los animales

En el género *Flavobacterium* no existen especies descritas como patógenas para el hombre, aunque se pueden encontrar en la literatura algunos casos de *Flavobacterium* spp. asociados a procesos respiratorios y sepsis, fundamentalmente en neonatos y pacientes inmunocomprometidos (Manfredi y col., 1999; Mosayebi y col., 2011). También se ha publicado un caso en el que se aisló por primera vez una cepa de *F. lindanitolerans* de una muestra de líquido ascítico de un paciente diagnosticado con una infección vírica, aunque la relevancia clínica de este aislamiento no ha sido dilucidada (Tian y col., 2011).

Por el contrario, en el ámbito veterinario si existen un importante grupo de especies patógenas de peces que afectan principalmente al cultivo de salmónidos en todo el mundo (Baudin-Laurencin y col., 1989; Bernardet y Kerouault, 1989). Estas bacterias dan origen a diversas patologías, desde necrosis ulcerativa de la piel hasta infecciones sistémicas (Wakabayashi y col., 1989; Holt y col., 1993; Shotts y Starliper, 1999; Starliper, 2011). Las especies de mayor significación clínica son *F. psychrophilum, F. columnare,* y *F. branchiophilum,* aunque otras especies del género, tales como *F. hydatis*, *F. johnsoniae*, *F. succinicans*, *Flavobacterium chilense*, *Flavobacterium araucananum* o *Flavobacterium spartansii* también se han aislado ocasionalmente de peces enfermos (Bernardet y col., 1996; Bernardet y Bowman, 2006; Flemming y col., 2007; Bernardet y Bowman, 2011; Kämpfer y col., 2012; Loch y Faisal, 2014b).

F. psychrophilum es el agente etiológico de dos patologías que causan una alta mortalidad en salmónidos: la enfermedad del agua fría (*bacterial cold water diseases*, BCWD) que afecta a peces adultos en los que ocasiona erosiones y ulceraciones en piel, necrosis de aletas y branquias y en los casos graves se observa descamación completa de la aleta caudal con exposición abierta de la espina en el área del pedúnculo caudal (Starliper, 2011), y el síndrome del alevín de la trucha arcoíris (*rainbow trout fry síndrome*, RTFS; Austin y Stobie, 1991) que es una enfermedad sistémica que provoca brotes severos durante la época invernal en salmónidos criados en piscifactorías a nivel mundial (Cipriano y col., 1995; Kondo y col., 2003; Nematollahi y col., 2003; Hesami y col., 2008). Esta patología afecta fundamentalmente a alevines que aún no son completamente inmunocompetentes, en los que cursa con un cuadro septicémico agudo con hipertrofia del bazo y elevada mortalidad. En algunos peces juveniles puede también observarse distensión abdominal y/o color anormalmente oscuro de la piel. La enfermedad normalmente tiene lugar cuando la temperatura del agua oscila entre 4 y 13 °C.

De igual forma, *F. columnare* es el responsable de la enfermedad de la columna, una enfermedad crónica que afecta a una amplia gama de peces de agua dulce a nivel mundial (Anderson y Conroy, 1969; Tien y col., 2012). Se presenta con lesiones externas que pueden, o no, llegar a ulcerarse; la bacteria suele adherirse a la superficie, las branquias y las aletas del pez provocando manchas blancas o áreas necróticas durante la infección activa, hasta que la colonización bacteriana conduce a la erosión de tejido (Wakabayashi, 1993). Los signos clínicos que frecuentemente presentan los peces afectados son erosión de la cola, manchas blancas en el cuerpo y branquias de color grisáceo (Shotts y Starliper, 1999; Pilarski y col., 2008). *F. columnare* no está asociado a infecciones sistémicas y normalmente no se recupera de órganos internos, aislándose más comúnmente de la superficie externa de los peces afectados (Foscarini, 1989; Austin y Austin, 2007; McElwain y col., 2009).

La especie *F. branchiophilum* es el agente causal de la enfermedad bacteriana de las branquias (*bacterial gill disease*, BGD). Tal como definió Wood (1974), el nombre de la enfermedad describe los signos clínicos de las infecciones bacterianas de las branquias. La enfermedad se caracteriza por altas tasas de morbilidad y mortalidad atribuibles a la colonización bacteriana masiva de las

branquias y las superficies laminares, además de la patología branquial progresiva derivada de las altas tasas de necrosis del epitelio laminar (Speare y col., 1995).

Las especies *F. johnsoniae, F. hydatis,* y *F. succinicans* han sido aisladas exclusivamente a partir de lesiones externas de peces, lo que sugiere que son probablemente organismos saprofitos o comensales que pueden colonizar las lesiones de peces iniciadas por otras bacterias o parásitos (Bernardet y Bowman, 2006).

Recientemente se han descrito tres especies del género a partir de salmónidos enfermos, *F. chilense* recuperada de lesiones externas de trucha arcoíris, *F. araucananum* aislada de riñon y lesiones externas de salmón del atlántico y *F. spartansii* aislada de riñon de salmón Chinook (Kämpfer y col., 2012; Loch y Faisal, 2014b). Otras cepas de *Flavobacterium* spp. saprofitas o comensales han sido encontradas en la superficie de huevos, piel, branquias, e intestino de peces (Huber y col., 2004; Bernardet y Bowman, 2006; Hu y col., 2007).

3.3 Género *Chryseobacterium*

3.3.1 Aspectos taxonómicos

El género *Chryseobacterium* se describió hace 20 años por Vandamme y col. (1994a). Inicialmente lo formaron seis especies (*Chryseobacterium balustinum*, *Chryseobacterium gleum*, *Chryseobacterium indologenes*, *Chryseobacterium indoltheticum*, [*Chryseobacterium*] *meningosepticum*, y *Chryseobacterium scophthalmum*), previamente incluidas en el género *Flavobacterium* y reclasificadas sobre la base de un estudio del 16S ARNr. *C. gleum* se consideró como la especie tipo del género por estar bien caracterizada y haber sido estudiada en detalle tanto fenotípica como genotípicamente por Holmes y col. (1984b).

Durante varios años no hubo cambios en el género *Chryseobacterium* y éste se mantuvo constituido sólo por las seis especies originales. No fue hasta el año 2003 cuando se añadieron dos nuevas especies al género, *Chryseobacterium joostei* (Hugo y col., 2003) y *Chryseobacterium defluvii* (Kämpfer y col., 2003). En el año 2004 se validó otra nueva especie, [*Chryseobacterium*] *miricola* (Li y col., 2003), aunque posteriormente fue transferida junto a [*C.*] *meningosepticum* al género *Elizabethkingia* (Kim y col., 2005b). A partir de ese momento se han

incorporado progresivamente distintas especies a este género. Así, en el año 2005 se describieron cinco nuevas especies, mientras que en 2006 y 2007 se añadieron al género 6 nuevas especies cada año. Solo en 2008 trece nuevas especies se sumaron al género, aunque una de las especies descritas, *Chryseobacterium arothri* (Campbell y col., 2008), fue reclasificada posteriormente por Kämpfer y col. (2009b) como sinónimo de *Chryseobacterium hominis* (Vaneechoutte y col., 2007) al no observarse diferencias sustanciales entre las cepas tipo de ambas especies después de un estudio comparativo en base a características genotípicas, fenotípicas y quimiotaxonómicas. En el año 2009, tres especies del género *Sejongia*, ([*Sejongia*] *antarctica*, [*Sejongia*] *jeonii* y [*Sejongia*] *marina*) pasaron a formar parte del género *Chryseobacterium* (Kämpfer y col., 2009a), la especie [*Kaistella*] *koreensis* fue reclasificada como *Chryseobacterium koreense* (Kämpfer y col., 2009c) y dos nuevas especies más fueron descritas. Desde entonces, cada año se han ido describiendo nuevas especies dentro del género (Figura 4) y en el momento de escribir esta memoria, octubre de 2014, el género lo constituyen 81 especies válidas (Euzeby y Parte, 2014a). Las especies que conforman el género, excepto las que se describen en este estudio, se enumeran en la Tabla 5.

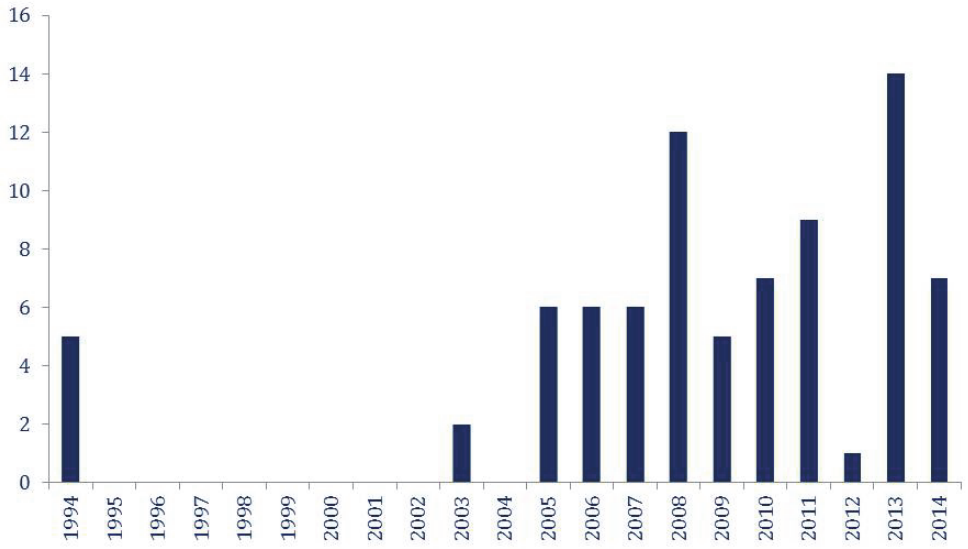

Figura 4. Número de especies válidamente publicadas en el género *Chryseobacterium* desde el año 1994 hasta octubre de 2014.

Tabla 5. Especies validadas del género *Chryseobacterium* indicando su origen y lugar de aislamiento

Especie	Fuente de aislamiento	Referencia
Chryseobacterium aahli	Riñón de trucha. USA.	Loch y Faisal, 2014a
Chryseobacterium angstadtii	Tanque de tritón. USA.	Kirk y col., 2013
Chryseobacterium antarcticum	Tierra. Antártida.	Kämpfer y col., 2009a
Chryseobacterium anthropi	Sangre humana. Bélgica.	Kämpfer y col., 2009b
Chryseobacterium aquaticum	Depósito de agua. Corea.	Kim y col., 2008
Chryseobacterium aquifrigidense	Agua refrigerada. Corea.	Park y col., 2008
Chryseobacterium arachidis	Rizosfera.	Kämpfer y col., 2014a
Chryseobacterium arthrosphaerae	Heces de Milpiés. India.	Kämpfer y col., 2010a
Chryseobacterium artocarpi	Suelo de la rizosfera deCempedak. Malasia	Venil y col., 2014
Chryseobacterium balustinum	Sangre de pez de agua dulce. Francia.	Vandamme y col., 1994
Chryseobacterium bernardetii	Esputo humano. Inglaterra.	Holmes y col., 2013
Chryseobacterium bovis	Leche de vaca cruda. Israel.	Hantsis-Zacharov y col., 2008a
Chryseobacterium caeni	Lodos de biorreactor. Corea.	Quan y col., 2007
Chryseobacterium camelliae	Hojas de té verde. Corea.	Kook y col., 2014
Chryseobacterium carnipullorum	Pollo crudo. Sudáfrica.	Charimba y col., 2013
Chryseobacterium carnis	Carne.	Holmes y col., 2013
Chryseobacterium chaponense	Salmon Atlántico enfermo. Chile.	Kämpfer y col., 2011
Chryseobacterium contaminans	Contaminante de una placa de agar. USA.	Kämpfer y col., 2014b
Chryseobacterium culicis	Intestino medio del mosquito Culex. India.	Kämpfer y col., 2010b

Tabla 5. Continuación

Especie	Fuente de aislamiento	Referencia
Chryseobacterium daecheongense	Sedimentos de lago de agua dulce. Corea.	Kim y col., 2005a
Chryseobacterium daeguense	Aguas residuales. Corea.	Yoon y col., 2007
Chryseobacterium defluvii	Aguas residuales. Alemania.	Montero-Calasanz y col., 2013
Chryseobacterium elymi	Rizosfera de plantas de dunas costeras. Corea.	Cho y col., 2011
Chryseobacterium flavum	Suelo contaminado con herbicidas. China.	Zhou y col., 2007
Chryseobacterium formosense	Rizosfera de lechuga de jardín. Taiwán.	Young y col., 2005
Chryseobacterium frigidisoli	Muestra de suelo (arena). Antártida.	Bajerski y col., 2013
Chryseobacterium gallinarum	Pollo. Alemania.	Kämpfer y col., 2014b
Chryseobacterium gambrini	Superficie de acero, planta embotelladora de cerveza. Alemania.	Herzog y col., 2008
Chryseobacterium geocarposphaerae	Rizosfera.	Kämpfer y col., 2014a
Chryseobacterium ginsengisoli	Rizosfera de ginseng. República de Corea.	Nguyen y col., 2013
Chryseobacterium ginsenosidimutans	Suelo. Corea.	Loch y Faisal, 2014a
Chryseobacterium gleum	Hisopo vaginal humano. Reino Unido.	Nguyen y col., 2013
Chryseobacterium greenlandense	Centro de núcleo de hielo. Groenlandia.	Loveland-Curtze y col., 2010
Chryseobacterium gregarium	Material vegetal en descomposición. Alemania.	Loch y Faisal, 2014a
Chryseobacterium gwangjuense	Suelo. Corea.	Park y col., 2013b
Chryseobacterium hagamense	Rizosfera de plantas de dunas costeras. Corea.	Cho y col., 2011

Tabla 5. Continuación

Especie	Fuente de aislamiento	Referencia
Chryseobacterium haifense	Leche cruda. Israel.	Hantsis-Zacharov y Halpern, 2007
Chryseobacterium hispalense	Estanque de agua de lluvia. España	Montero-Calasanz y col., 2013
Chryseobacterium hispanicum	Agua potable. España.	Gallego y col., 2006
Chryseobacterium hominis	Sangre humana. Bélgica.	Vaneechoutte y col., 2007
Chryseobacterium humi	Sedimentos contaminados industrialmente. Portugal.	Pires y col., 2010
Chryseobacterium hungaricum	Suelos contaminados con hidrocarburos. Hungría.	Szoboszlay y col., 2008
Chryseobacterium indologenes	Tráquea humana. EEUU.	Montero-Calasanz y col., 2013
Chryseobacterium indoltheticum	Lodo marino.	Wu y col., 2013
Chryseobacterium jejuense	Suelo. República de Corea.	Wu y col., 2013
Chryseobacterium jeonii	Tierra. Antártida.	Kämpfer y col., 2009a
Chryseobacterium joostei	Leche de vaca cruda. Sudáfrica.	Hugo y col., 2003
Chryseobacterium koreense	Agua dulce. Corea.	Kämpfer y col., 2009
Chryseobacterium kwangjuense	Raíz de planta de pimiento. Corea.	Sang y col., 2013
Chryseobacterium lactis	Enjuague de botella de leche. Reino unido.	Holmes y col., 2013
Chryseobacterium lathyri	Rizosfera de plantas de dunas costeras. Corea.	Cho y col., 2011
Chryseobacterium luteum	Pastos. Alemania.	Behrendt y col., 2007
Chryseobacterium marinum	Agua de mar. Antártida.	Kämpfer y col., 2009a

Tabla 5. Continuación

Especie	Fuente de aislamiento	Referencia
Chryseobacterium molle	Biofilm, planta de embotellado de cerveza. Alemania.	Herzog y col., 2008
Chryseobacterium nakagawai	Absceso renal humano. Reino Unido	Holmes y col., 2013
Chryseobacterium oranimense	Leche de vaca cruda. Israel.	Hantsis-Zacharov y col., 2008b
Chryseobacterium pallidum	Superficie de acero, planta embotelladora de cerveza. Alemania.	Herzog y col., 2008
Chryseobacterium palustre	Sedimento contaminado industrialmente. Portugal.	Pires y col., 2010
Chryseobacterium piperi	Sedimentos y agua de un arroyo. USA.	Strahan y col., 2011
Chryseobacterium piscicola	Salmón del atlántico enfermo. Chile.	Ilardi y col., 2009
Chryseobacterium piscium	Pescado fresco. Sur del océano Atlántico. Sudáfrica.	de Beer y col., 2006
Chryseobacterium rhizosphaerae	Rizosfera de plantas de dunas costeras. Corea.	Cho y col., 2011
Chryseobacterium rigui	Humedal estuarino del río Han. Corea.	Park y col., 2013a
Chryseobacterium scophthalmum	Branquias de rodaballo enfermo. Escocia.	Vandamme y col., 1994
Chryseobacterium shigense	Bebida de ácido láctico. Japón.	Shimomura y col., 2005
Chryseobacterium soldanellicola	Raíz de planta de duna de arena. Corea.	Park y col., 2006
Chryseobacterium soli	Muestra de suelo. República de Corea.	Weon y col., 2008

Tabla 5. Continuación

Especie	Fuente de aislamiento	Referencia
Chryseobacterium solincola	Suelo contaminado con hidrocarburos. Argelia.	Benmalek y col., 2010
Chryseobacterium taeanense	Raíz de planta de duna de arena. Corea.	Park y col., 2006
Chryseobacterium taichungense	Suelo contaminado con alquitrán. Taiwán.	Shen y col., 2005
Chryseobacterium taihuense	Espuma de algas en descomposición. Lago Taihu, China.	Wu y col., 2013
Chryseobacterium taiwanense	Suelo. Taiwán.	Wu y col., 2013
Chryseobacterium taklimakanense	Suelo del desierto. China.	Holmes y col., 2013
Chryseobacterium treverense	Sangre humana. Alemania.	Yassin y col., 2010
Chryseobacterium ureilyticum	Superficie de acero, planta embotelladora de cerveza. Alemania.	Herzog y col., 2008
Chryseobacterium vietnamense	Suelo forestal. Vietnam.	Li y Zhu, 2012
Chryseobacterium vrystaatense	Pollo crudo. Sudáfrica.	De Beer y col., 2005
Chryseobacterium wanjuense	Suelo de invernadero cultivado con lechuga. República de Corea.	Montero-Calasanz y col., 2013.
Chryseobacterium xinjiangense	Permafrost alpino (suelo). China.	Zhao y col., 2011
Chryseobacterium yonginense	Lago artificial. República de Corea.	Joung y Joh, 2011
Chryseobacterium zeae	Rizosfera.	Kämpfer y col., 2014a

La información detallada, incluyendo sinónimos, datos históricos de los nombres, cepas tipo, etc., puede obtenerse a través de la *List of Prokaryotic Names with Standing in Nomenclature*, disponible en la dirección http://www.bacterio.net/chryseobacterium.html (Euzeby y Parte, 2014a). En aquellos nombres derivados de nuevas combinaciones o cuyas descripciones han sido corregidas solo se muestra la referencia más reciente.

3.3.2 Características fenotípicas, genotípicas y quimiotaxonómicas

Los miembros del género *Chryseobacterium* son bacilos Gram-negativos, inmóviles, que no forman esporos, con lados paralelos y extremos redondeados, que generalmente tienen un tamaño aproximado de 0,5 µm de ancho y una longitud variable, a menudo entre 1-3 µm (Vandamme y col., 1994a). No obstante, existen en el género algunas especies singulares, como *Chryseobacterium shigense* (Shimomura y col., 2005) y *Chryseobacterium marinum* (Kämpfer y col., 2009a) descritas como bacilos ligeramente curvados y la especie *Chryseobacterium daeguense* (Yoon y col., 2007) en cuya descripción se indica que algunas células son ovales o cocoides.

Son microorganismos quimiorganótrofos, con un tipo de metabolismo estrictamente aerobio con el oxígeno como aceptor final de electrones (Vandamme y col., 1994a; Bernardet y col., 2002). Sin embargo, algunas especies del género como *C. scophthalmum*, *Chryseobacterium bovis*, *Chryseobacterium antarcticum*, *Chryseobacterium jeonii*, *Chryseobacterium taihuense* y algunas cepas de *C. gleum* y *C. indologenes,* son capaces de crecer en condiciones anaerobias (Yabuuchi y col., 1983; Holmes y col., 1984a, b*;* Mudarris y col., 1994; Hantsis-Zacharov y col., 2008a; Kämpfer y col., 2009a; Wu y col., 2013).

El rango de temperatura al que son capaces de crecer estos microorganismos es muy amplio. Según la descripción original del género, basándose en las seis especies iniciales (Vandamme y col., 1994a) todos sus miembros crecen a 30 °C y la mayoría a 37 °C. Sin embargo, muchas de las especies descritas posteriormente muestran un crecimiento psicrotolerante a 5 °C, todas crecen de 15 a 30 °C, y unas pocas pueden crecer a la temperatura de 42 °C (Bernardet y col., 2006). También se han encontrado algunas cepas pertenecientes al género capaces de crecer a temperaturas más bajas como por ejemplo una cepa de *Chryseobacterium* spp., aislada del suelo de un bosque en el norte de Finlandia (Männistö y Häggblom, 2006) y la especie *Chryseobacterium frigidisoli*, aislada de un glaciar en la Antártida (Bajerski y col., 2013), ambas capaces de crecer a 0 °C.

En los medios sólidos comúnmente usados para el cultivo (agar sangre, agar tripticasa soja, R2A y agar nutritivo), estos microorganismos forman colonias circulares, convexas, de translúcidas a opacas, lisas y brillantes con bordes enteros que emanan un olor fuerte y aromático. Por lo general, las colonias son pigmentadas de color amarillo-naranja debido a la producción de pigmentos del

tipo flexirrubina (Bernardet y col., 2002), aunque existen también algunas especies, como *C. hominis* (Vaneechoutte y col., 2007) y *Chryseobacterium palustre* (Pires y col., 2010) que no producen este pigmento y otras como *Chryseobacterium yonginense* (Joung y Joh, 2011), *Chryseobacterium haifense* (Hantsis-Zacharov y Halpern, 2007) y *C. bovis* (Hantsis-Zacharov y col., 2008a) que presentan, además del pigmento flexirrubina, pigmentos del tipo carotenoide. Algunas especies del género cuando son cultivadas en medio agar tirosina producen un pigmento difusible de color rosado-marrón a marrón oscuro (Mudarris y col., 1994; Hugo y col., 2003), aunque esta característica no se ha investigado en todas las especies del género.

Otras características comunes a la mayoría de los microorganismos que conforman este género son la producción de las enzimas catalasa, oxidasa y fosfatasa, su fuerte actividad proteolítica, su capacidad para oxidar distintos carbohidratos incluyendo el glicerol y la trehalosa, y para hidrolizar la esculina pero no el agar y su resistencia a una amplia gama de agentes antimicrobianos (Vandamme y col., 1994a; Bernardet y col., 2006).

Para la diferenciación de las especies pertenecientes al género *Chryseobacterium* se recomiendan una serie de pruebas fenotípicas entre las que se encuentran: crecimiento en agar cetrimida y agar MacConkey, crecimiento a 5, 37 y 42 °C, producción de ácido a partir de diferentes azúcares, reducción de nitratos y nitritos, producción de H_2S e indol y de las enzimas L-fenilalanina-diaminasa, ureasa, y β-galactosidasa, formación de precipitado en agar yema de huevo al 10% y la hidrolisis de almidón, Tween 80 y L-tirosina (Bernardet y col. 2002).

Desde el punto de vista genético, el contenido G+C del ADN de los miembros del género varía en el rango de 33-38 mol% (Vandamme y col., 1994a). Sin embargo, algunas especies descritas con posterioridad a la creación del género presentan valores inferiores, como es el caso de *Chryseobacterium soldanellicola* y *Chryseobacterium taeanense* (28,8 y 32,1 mol%, respectivamente; Park y col., 2006b), *Chryseobacterium piscicola* (32,5 mol%; Ilardi y col., 2009) y *C. yonginense* (31,3 mol%; Joung y Joh, 2011).

Desde el punto de vista quimiotaxonómico, las especies que conforman el género, al igual que el resto de miembros de la familia *Flavobacteriaceae*, contienen MK-6 como única o principal quinona respiratoria y no presentan

esfingofosfolípidos. En cuanto al perfil de ácidos grasos todas las especies de *Chryseobacterium* contienen los mismos ácidos grasos en proporciones bastante similares, siendo los ácidos grasos predominantes iso-$C_{15:0}$, iso-$C_{17:0}$ 3-OH, iso-$C_{17:1}$ *ω9c*, y *summed feature* 4 (que comprende iso-$C_{15:0}$ 2-OH y/o $C_{16:1}$ *ω7c/t*). Esto permite diferenciar las especies del género *Chryseobacterium* de otros géneros de la familia *Flavobacteriaceae* (por ejemplo, *Elizabethkingia*, *Empedobacter*, *Riemerella* y *Bergeyella*) que muestran composiciones de ácidos grasos bastante distintas (Hugo y col., 1999; Bernardet y col., 2006).

3.3.3 Ecología

Como se puede observar en la Tabla 5, las especies de *Chryseobacterium* están presentes en una gran variedad de hábitats que van desde entornos industriales, ambientes naturales, muestras clínicas y alimentos.

Entre las fuentes ambientales de las que se han recuperado microorganismos de este género se encuentran los lodos marinos y el agua de mar (Campbell y Williams, 1951; Lee y col., 2007), hielo glaciar (Loveland-Curtze y col., 2010), sistema de refrigeración de agua (Park y col., 2008), fuentes de agua dulce y sedimentos de lago (Kim y col., 2005a, 2008; Joung y Joh, 2011; Strahan y col., 2011;), agua potable (Gallego y col., 2006), suelo (Yi y col., 2005b; Weon y col., 2006; Zhou y col., 2007; Szoboszlay y col., 2008; Benmalek y col., 2010; Im y col., 2011; Li y Zhu, 2012), la rizosfera de diversas plantas (Young y col., 2005; Park y col., 2006b; Cho y col., 2010), pastos (Behrendt y col., 2007) y material vegetal en descomposición (Behrendt y col., 2008).

En los entornos industriales se han aislado diferentes especies de *Chryseobacterium* de lodos de biorreactor (Quan y col., 2007), aguas residuales (Kämpfer y col., 2003; Yoon y col., 2007), sedimentos contaminados industrialmente (Pires y col., 2010) y de superficies de acero en plantas de embotellado de cerveza (Herzog y col., 2008).

Entre los alimentos donde se han aislado especies de *Chryseobacteriun* se encuentran los productos lácteos, como leche de vaca cruda (Hugo y col., 2003; Hantsis-Zacharov y Halpern, 2007; 2008a, b) y una bebida de ácido láctico (Shimomura y col., 2005), así como carne y productos de aves de corral (Hayes, 1977; García-López y col., 1998; De Beer y col., 2005) o pescado (Gennari y Cozzolino, 1989; Morita y col., 1997; González y col., 2000; Lijnen y col., 2000; De

Beer y col., 2006). Aunque en algunas casos estos microorganismos forman parte de la comunidad bacteriana normal del alimento del que fue aislado, como por ejemplo *C. shigense* (Shimomura y col., 2005), en otras ocasiones la presencia de estos microorganismos está relacionada con el deterioro de la leche y los productos lácteos (Venter y col., 1999) y del pescado (Harrison, 1929; Gennari y Cozzolino, 1989; Engelbrecht y col., 1996a, b).

3.3.3.1 Especies relacionadas con procesos clínicos en el hombre y los animales

Algunos microorganismos del género se han asociado con procesos clínicos en humanos. Por lo general, se considera que poseen una baja virulencia y son considerados patógenos oportunistas causantes de infecciones nosocomiales principalmente en recién nacidos y pacientes inmunocomprometidos (Bernardet y col., 2005). Aislados de *Chryseobacterium* spp. se obtienen con frecuencia de entornos hospitalarios en los que son capaces de colonizar la superficie de distintos instrumentos y equipos médicos (Lu y Chan, 1997; Schreckenberger y col., 2003), siendo su utilización el origen de las infecciones en las personas (Holmes y Owen, 1981; Bernardet y col., 2006). Entre las especies del género con significación clínica en el hombre se encuentran *C. hominis* (Vaneechoutte y col., 2007), *Chryseobacterium anthropi* (Kämpfery col., 2009b) y *Chryseobacterium treverense* (Yassin y col., 2010) aisladas de muestras de sangre humana; *C. gleum* recuperado de diferentes muestras clínicas vaginales, fluido cerebroespinal, etc. (Holmes y col., 1984b); y *C. indologenes* que se ha relacionado con diferentes tipos de infecciones, tales como bacteriemia (Douvoyiannis y col., 2010; Ceylan y col., 2011; Chen y col., 2012a; Degandt y col., 2013), neumonía (Chen y col., 2012a), meningitis (Hendaus y Zahraldin, 2013; Olbrich y col., 2013), e infecciones oculares (Lu y Chan, 1997; Doiz y col., 1999; Ramos-Esteban y col., 2008). Recientemente se ha incorporado al género una nueva especie, *Chryseobacterium bernardetii* (Holmes y col., 2013) aislada de una muestra de esputo humano.

En el ámbito veterinario, los microorganismos del género *Chryseobacterium* no son patógenos relevantes para los animales domésticos. Sin embargo, algunas especies han sido asociadas con enfermedades en peces marinos y de agua dulce. Una de estas especies es *C. balustinum*, aislada de la sangre de un pez de agua dulce enfermo que mostraba signos de septicemia hemorrágica (Harrison, 1929). También la especie *C. scophthalmum* se ha asociado

con casos de hiperplasia branquial y septicemia hemorrágica en rodaballo (Mudarris y Austin, 1989; Mudarris y col., 1994; Bernardet y col., 2006). De igual forma, *C. joostei*, se ha aislado tanto de lesiones externas como de órganos internos en diferentes especies de peces enfermos (Bernardet y col., 2005). Otras especies, descritas más recientemente y relacionadas igualmente con peces enfermos son *C. arothri* (actualmente *C. hominis;* Kämpfer y col., 2009b) aislado del riñón de un pez globo (Campbell y col., 2008), *C. piscicola*, que se aisló de lesiones externas de salmónidos enfermos (salmón del atlántico y trucha arcoíris) (Ilardi y col., 2009) y *Chryseobacterium chaponense* aislado de lesiones externas, branquias y aleta de salmones enfermos del atlántico (Kampfer y col., 2011).

Objetivos

4 Objetivos

Los miembros que conforman la familia *Flavobacteriaceae*, entre ellos los géneros *Flavobacterium* y *Chryseobacterium,* son microorganismos ubicuos que habitan una amplia variedad de hábitats terrestres y acuáticos (Bernardet y Bowman, 2006; Bernardet y Nakagawa, 2006; Bernardet y col., 2006). La mayoría de los miembros de ambos géneros son saprófitos, aunque algunas especies tienen significación clínica en el ámbito veterinario, fundamentalmente como patógenos de peces (Shotts y Starliper, 1999; Austin y Austin, 2007). Así, las especies *F. psychrophilum, F. columnare*, y *F. branchiophilum* son importantes patógenos de peces que afectan principalmente al cultivo de salmónidos en todo el mundo, siendo responsables de diversas patologías, desde necrosis ulcerativa de la piel hasta infecciones sistémicas (Baudin-Laurencin y col., 1989; Bernardet y Kerouault, 1989; Holt y col., 1993; Austin y Austin, 2007). Otras especies de ambos géneros como *F. johnsoniae* (Suebsing y Kim 2012), *F. succinicans* (Anderson y Ordal, 1961), *F. hydatis* (Strohl y Tait, 1978), *F. chilense*, y *F. araucananum* (Kämpfer y col., 2012), *F. spartansii* (Loch y Faisal, 2014b), *C. piscium* (De Beer y col., 2006), *C. piscicola* (Ilardi y col., 2009), *C. arothri* y *C. chaponense* (Kämpfer y col., 2009b, 2011), *C. balustinum* (Harrison, 1929), *C. scophthalmum* (Mudarris y Austin, 1989; Mudarris y col., 1994; Bernardet y col., 2006) y *C. joostei* (Bernardet y col., 2005) también se han aislado ocasionalmente de peces enfermos. Este aumento en el número de especies asociadas con ciertos procesos clínicos en peces ha hecho que los miembros de los géneros *Flavobacterium* y *Chryseobacterium* se consideren patógenos potenciales de peces con una significación clínica emergente (Bernardet y col., 2005; Michel y col., 2005; Austin y Austin, 2007; Loch y col., 2014).

Durante el periodo comprendido entre mayo de 2008 y julio de 2009, se remitieron a nuestro laboratorio una serie de alevines enfermos de trucha arcoíris, procedentes de varios episodios clínicos, que presentaban características clínicas compatibles con una infección por *F. psychrophilum*, así como algunos huevos embrionados. A partir de distintos órganos de diferentes alevines y en tres muestras de huevos embrionados se aislaron en medio *Anacker and Ordal* una serie de bacterias Gram negativas, ninguna de las cuales pudo identificarse como *F. psychorphilum* mediante una PCR específica para este patógeno (Wiklund y col., 2000). Dado que los microorganismos fueron aislados mayoritariamente de alevines enfermos, en algunos casos en

cultivo puro, podrían tener una significación clínica en el proceso infeccioso que padecían los alevines y por tanto consideramos interesante su identificación.

Teniendo en cuenta estos antecedentes, en el presente trabajo de Tesis Doctoral nos propusimos como objetivo realizar el estudio taxonómico polifásico de los microorganismos aislados integrando datos filogenéticos, genotípicos, fenotípicos y de caracterización molecular con el fin de esclarecer la posición taxonómica de estos microorganismos así como establecer criterios útiles para su identificación. Para conseguir este objetivo realizamos las siguientes tareas de investigación:

- Análisis filogenético basado en la comparación de las secuencias del gen que codifica para el 16S ARNr.
- Estudio genotípico mediante estudios de hibridación ADN-ADN y análisis del contenido G+C del ADN.
- Análisis de características quimiotaxonómicas.
- Caracterización fenotípica a través del estudio de características fisiológicas y bioquímicas.
- Caracterización de los aislados mediante técnicas de tipificación molecular.

Resultados

5 Resultados

5.1 Caracterización taxonómica de microorganismos del género *Flavobacterium*

El género *Flavobacterium* es un género muy antiguo descrito por primera vez por Bergey y col. (1923) para englobar un grupo muy heterogéneo de bacterias pigmentadas de color amarillo. Mayoritariamente lo constituyeron bacterias Gram negativas, inmóviles o móviles con flagelo polar o peritricos, así como algunas especies Gram positivas, quedando el género formado inicialmente por 46 especies.

A lo largo de su historia el género *Flavobacterium* ha sufrido numerosas modificaciones y reclasificaciones (Bergey y col., 1939; Weeks y Breed, 1957; Weeks, 1974; Holmes y Owen, 1979; Holmes y col., 1984a). No fue hasta el año 1996 cuando Bernardet y col. realizaron un extenso estudio polifásico que permitió una clara delimitación de las especies incluidas en este género. Como consecuencia de este estudio el género quedó formado por diez especies: *F. aquatile*, *F. branchiophilum, Flavobacterium columnare, F. flevense, F. hydatis, F. johnsoniae, F. pectinovorum, F. psychrophilum, F. saccharophilum* y *F. succinicans*. Desde entonces, el género *Flavobacterium* se ha expandido considerablemente debido a la descripción de muchas nuevas especies, estando formado en la actualidad, octubre de 2014 por 107 especies válidamente publicadas (Euzeby y Parte, 2014c).

Los microorganismos que conforman el género se pueden aislar a partir de diferentes fuentes tales como agua dulce, suelo, ambientes marinos y salinos, peces, lodos activados, materia vegetal en descomposición, entre otras (Euzeby y Parte, 2014c). Algunas especies del género como *F. psychrophilum, F. columnare* y *F. branchiophilum* son importantes patógenos de peces que afectan principalmente al cultivo de salmónidos en todo el mundo (Baudin-Laurencin y col., 1989; Bernardet y Kerouault, 1989). Además de estas tres especies, otras como *F. hydatis*, *F. johnsoniae*, *F. succinicans*, *F. chilense*, *F. araucananum* o *F. spartansii* también se han aislado ocasionalmente de peces enfermos (Bernardet y col., 1996; Bernardet y Bowman, 2006; Flemming y col., 2007; Bernardet y Bowman, 2011; Kämpfer y col., 2012; Loch y Faisal, 2014b). Estos microorganismos, dependiendo de la especie, dan origen a diversas patologías, desde necrosis ulcerativa de la piel hasta infecciones sistémicas

(Wakabayashi y col., 1989; Holt y col. 1993; Shotts y Starliper, 1999; Starliper, 2011).

En este capítulo se estudian una serie de aislados pertenecientes al género *Flavobacterium* recuperados a partir de alevines de trucha arcoíris enfermos que presentaban síntomas clínicos compatibles con una infección por *F. psychrophilum*, así como tres aislados recuperados de huevos embrionados. Los aislados se obtuvieron en medio *Anacker and Ordal* comúnmente empleado para el aislamiento de *F. psycrophilum*, en el que crecieron formado colonias de color amarillo. Sin embargo, microscópicamente estos microorganismos no presentaron la típica morfología celular de bacilos filamentosos que muestra *F. psychrophilum* (Cipriano y Holt, 2005) y tampoco pudo confirmarse la identificación como *F. psychrophilum* utilizando una PCR específica para este patógeno (Wiklund y col., 2000).

Con el fin de establecer la posición taxonómica de estos microorganismos realizamos un amplio estudio taxonómico polifásico que incluyó el análisis filogenético y la caracterización bioquímica, fisiológica y quimiotaxonómica de los aislados. Además se realizaron estudios de hibridación ADN-ADN y del contenido en G+C del ADN. El análisis de las secuencias del 16S ARNr de los aislados mostró que estos se distribuían formando siete grupos filogenéticos (F1-F7) que presentaban porcentajes de similitud en la secuencia de dicho gen mayores del 98,9% con diferentes especies del género *Flavobacterium.* Los estudios de hibridación ADN-ADN entre los aislados de los grupos F1-F7 y las especies filogenéticamente más próximas mostraron porcentajes de hibridación que oscilaron entre 11,0 y 56,3%, valores claramente inferiores al porcentaje del 70% de homología considerado como mínimo para asignar dos taxones a la misma especie (Wayne y col., 1987). El contenido en G+C del ADN varió entre 33,0 y 36,2 mol%, valores que están dentro del rango descrito para el género *Flavobacterium* (Bernardet y Bowman, 2010). Estos resultados permitieron la descripción formal de siete nuevas especies del género *Flavobacterium* para las cuales se propusieron los nombres específicos: *Flavobacterium oncorhynchi* sp. nov. (grupo F1), *Flavobacterium plurextorum* sp. nov. (grupo F2), *Flavobacterium tructae* sp. nov. (grupo F3), *Flavobacterium piscis* sp. nov. (grupo F4), *Flavobacterium collinsii* sp. nov. (grupo F5), *Flavobacterium branchiarum* sp. nov. (grupo F6) y *Flavobacterium branchiicola* sp. nov. (grupo F7).

Todas las especies presentaron MK-6 como principal quinona respiratoria. En relación a los diferentes ácidos grasos, aunque existieron diferencias cuantitativas en el porcentaje de cada uno de los ácidos grasos detectados entre las diferentes especies, todas ellas mostraron iso-$C_{15:0}$ como ácido graso mayoritario, observándose niveles entre 19-33%. La fosfatidiletanolamina fue el principal lípido polar en todas las especies estudiadas. Los resultados quimiotaxonómicos obtenidos coinciden con los descritos para los miembros del género *Flavobacterium* (Bernardet y col., 2002; Bernardet y Nakagawa, 2006; Bernardet y Bowman, 2011) y confirman la adscripción de las nuevas especies al género *Flavobacterium* basada en el estudio filogenético.

Las nuevas especies pueden distinguirse por pruebas como la degradación de la L-tirosina (positiva para las especies *F. plurextorum*, *F. tructae*, *F. collinsii* y *F. branchiicola* y negativa para *F. oncorhynchi, F. piscis* y *F. branchiarum*), la degradación de la urea (únicamente positiva para *F. branchiicola*), la formación de pigmento difusible marrón en agar L-tirosina (observado solo en la especie *F. oncorhynchi*) y la actividad β-galactosidasa (ausente solamente en *F. branchiarum*). Igualmente pueden diferenciarse de las especies filogenéticamente más próximas.

El presente estudio ha contribuido a tener un mejor conocimiento de la diversidad de especies del género *Flavobacterium* relacionadas con infecciones en peces. Además, se describen una serie de pruebas que pueden ayudar a la correcta diferenciación de las especies descritas tanto de las tres especies patógenas de peces, *F. psychrophilum, F. columnare,* y *F. branchiophilum,* como de las distintas especies del género asociadas a procesos clínicos en peces.

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Flavobacterium oncorhynchi sp. nov., a new species isolated from rainbow trout (*Oncorhynchus mykiss*)☆

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ABSTRACT

Eighteen isolates of a Gram-negative, catalase and oxidase-positive, rod-shaped bacterium, recovered from diseased rainbow trout (*Oncorhynchus mykiss*), were characterized, using a polyphasic taxonomic approach. Studies based on comparative 16S rRNA gene sequence analysis showed that that the eighteen new isolates shared 99.2–100% sequence similarities. Phylogenetic analysis revealed that isolates from trout belonged to the genus *Flavobacterium*, showing the highest sequence similarities to *F. chungangense* (98.6%), *F. frigidimaris* (98.1%), *F. hercynium* (97.9%) and *F. aquidurense* (97.8%). DNA–DNA reassociation values between the trout isolates (exemplified by strain 631-08^T) and five type strains of the most closely related *Flavobacterium* species exhibited less than 27% similarity. The G + C content of the genomic DNA was 33.0 mol%. The major respiratory quinone was observed to be menaquinone 6 (MK-6) and iso-$C_{15:0}$, $C_{15:0}$ and $C_{16:1}$ $\omega7c$ the predominant fatty acids. The polar lipid profile of strain 631-08^T consisted of phosphatidylethanolamine, unknown aminolipids AL1 and AL3, lipids L1, L2, L3 and L4 and phospholipid PL1. The novel isolates were differentiated from related *Flavobacterium* species by physiological and biochemical tests. On the basis of the evidence from this polyphasic study, it is proposed that the isolates from rainbow trout be classified as a new species of the genus *Flavobacterium*, *Flavobacterium oncorhynchi* sp. nov. The type strain is 631-08^T (=CECT 7678^T = CCUG 59446^T).

Introduction

The genus *Flavobacterium* accommodates Gram-negative, non-spore-forming, aerobic, oxidase-positive, non-fermenting, predominantly gliding, yellow-pigmented bacteria that have menaquinone-6 as the primary respiratory quinone [1,4]. The genus has undergone a considerable expansion in the last decade; currently the genus *Flavobacterium* comprises 75 species with validly published names at the time of writing [11]. Due to their physiological diversity, they have been isolated from a range of ecological niches, including freshwater and saltwater ecosystems [4]. A number of *Flavobacterium* spp. are pathogenic or are regarded as opportunistic pathogens, causing disease in a wide variety of organisms, including plants, fish and humans [4,16,19]. One of the major concerns regarding some members of this genus is their ability to cause disease in aquaculture settings. Thus, several species, including *Flavobacterium psychrophylum*, *Flavobacterium johnsoniae*, *Flavobacterium branchiophilum* and *Flavobacterium columnare* have been associated with clinical diseases in fishes and represent major threats to commercial aquaculture worldwide [4,8,12,13]. Other species of *Flavobacterium* such as *Flavobacterium aquatile*, *Flavobacterium hydatis* and *Flavobacterium succinicans* also are isolated occasionally from diseased fish [1,4,9].

In the present study, identification of isolates, recovered during the period of 2008–2009, from diseased juvenile rainbow trout (*Oncorhynchus mykiss*) presenting clinical symptoms indicative of *F. psychrophylum* infection was accomplished using phenotypic and genotypic techniques.

Materials and methods

Isolation, morphological, physiological and biochemical characteristics

Diseased trout were sent (alive) to the Centro Visavet at the Veterinary School in Madrid, Spain, for routine bacteriological analysis.

☆ The GenBank accession number for the 16S rRNA gene sequence of strains 631-08^T, 646B-08 and 666-09 are FN669776, FR870076 and FR870077, respectively.

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Samples from internal organs (spleen, liver, and kidney) and gills were spread plated on Anacker and Ordal agar [4] and incubated at 22 °C for 7 days. Bacterial colonies displaying a yellow color, characteristic for flavobacteria, were selected for further analysis. Eighteen Gram-negative rod-shaped organisms were recovered from the liver (628-1-08, 631-08^T, 646-08, 650-08, 662-09, 666-09) and gills (425B-08, 426B-08, 433B-08, 437B-08, 441B-08, 646B-08, 688B-08, 695B-08, 947B-08, 950B-08, 22B-09, 47B-2-09). The yellow pigmented colonies of the isolates on Anacker and Ordal agar [4] and their Gram-staining characteristic were consistent with the presumptive diagnosis of infection by *F. psychrophylum*. However, attempts to confirm the identification of the bacteria from the diseased trout as *F. psychrophilum*, using a species-specific PCR assay [31], were unsuccessful; none of the 18 isolates gave the expected amplicon of 1089 bp, characteristic of this bacterial fish pathogen. For further studies, isolates were cultured on tryptone glucose extract agar (Difco) and incubated at 22 °C for 72 h under aerobic conditions. Stock cultures of the isolates were stored at −40 °C.

The standards for the description of new taxa in the family *Flavobacteriaceae* were followed for phenotypic characterization [2]. A number of key characteristics were performed, using standard procedures [5,6,27], i.e., Gram-staining, production of catalase and oxidase, and hydrolysis of agar, casein, L-tyrosine, aesculin, DNA, urea, gelatin and starch. Growth in brain heart infusion broth was assessed at 15, 25, 30, 37 and 42 °C, with 3.0, 4.5 and 6.5% added NaCl, and under anaerobic (with 4–10% CO_2) and microaerobic (with 5–15% O_2 and 5–12% CO_2) conditions, using GasPak Plus and CampyPak Plus sytems (BBL), respectively. Growth was tested on MacConkey (bioMérieux), nutrient (Difco), trypticase-soy (bioMérieux) and marine (Difco) agars. The presence of gliding motility and the production of flexirubin-type pigments and extracellular glycans were investigated as described previously [2]. The strains were further biochemically characterized, using the API 20NE systems (bioMérieux) according to the manufacturer's instructions, except that the incubation temperature for API 20NE was 22 °C. The type strains of species *Flavobacterium chungangense* CCUG 58910^T, *Flavobacterium frigidimaris* CCUG 59364^T, *Flavobacterium hercynium* CCUG 59448^T, *Flavobacterium aquidurense* CCUG 59847^T and *Flavobacterium resistents* CCUG 59848^T were used as reference strains for the investigation of the phenotypic properties of trout isolates under the same laboratory conditions.

Isolate 631-08^T has been deposited in the Spanish Type Culture collection (CECT) and in the Culture Collection of the University of Gothenburg (CCUG) Sweden, under the accession numbers CECT 7678^T and CCUG 59446^T, respectively. Isolates 666-09 and 646-08 were also deposited in the CECT and CCUG under the accession numbers CECT 7848 and CCUG 59447 and CECT 7792 and CCUG 59310, respectively

Cell fatty acid compositions, respiratory quinone and polar lipids analyses

Strains 631-08^T (CCUG 59446^T), 646-08 (CCUG 59310) and 666-09 (CCUG 59447) and the type strains of species analysed for comparison (Table 1) were cultivated aerobically, on Columbia II agar base (BBL 4397596) with 5% horse blood, at 30 °C, and harvested after 30 h ± 2 h. Cell fatty acid-fatty acid methyl ester (CFA-FAME) analyses were performed, using a protocol similar to that of the MIDI Sherlock MIS system [26]. Bacterial biomass was removed from the agar medium using a plastic inoculating loop, carefully scraped to avoid including medium in the sample; 50–100 mg of cells were transferred to glass tubes. The cells were saponified by mild alkaline methanolysis and released fatty acids were methylated followed by organic extraction CFAs were identified and quantified by gas-chromatography (Hewlett Packard HP 5890). Retention times of CFA peaks were converted to Equivalent Chain Length (ECL) values and the % area for each peaks was determined. The Agilent MIS FAME standard was used as reference for identification of peaks. The relative amount of each CFA in a strain was expressed as a percentage of the total fatty acids in the profile of a strain. Further details of the methodology can be found at http://www.ccug.se/pages/CFA_method_2008.pdf.

Determination of the respiratory quinone and polar lipids of the type strain (631-08^T) was carried out by the identification service of the DSMZ and Dr. B.J. Tindall (DSMZ, Braunschweig, Germany).

16S rRNA gene sequencing, DNA–DNA hybridization and DNA G+C content

The phylogenetic affinity of the isolates was established by sequencing of their 16S rRNA gene, as described previously [29]. A continuous segment (approximately 1395 bases) of the 16S rRNA gene of three isolates (433B-08 and 441B-08 and 631-08^T) and 1000 nucleotides from the other fifteen isolates were determined from PCR-amplified products, derived from universal primers pA (5′-AGAGTTTGATCCTGGCTCAG; positions 8-27, *Escherichia coli* numbering) and pH* (5′-AAGGAGGTGATCCAGCCGCA; positions 1541–1522. Sequence searches of GenBank were performed, using the program FASTA [23]. Sequences of the most closely related species and other representative species within the genus *Flavobacterium* were retrieved from GenBank and aligned with the newly determined sequences, using the program, SeqTools [23]. Phylogenetic trees were constructed according to three different algorithms: Neighbour-Joining [25], using the programs SeqTools and TREEVIEW [22,24], Maximum-Likelihood, using the PHYML software [14]; and Maximum-Parsimony, using the software package MEGA (Molecular Evolutionary Genetics Analysis) version 4 [18]. Genetic distances for the Neighbour-Joining and the Maximum-Likelihood algorithms were calculated by the Kimura two-parameter [17] and close-neighbour-interchange (search level = 2, random additions = 100) was applied in the Maximum-Parsimony analysis. The stabilities of the groupings were estimated by bootstrap analysis (1000 replications).

DNA–DNA hybridization experiments were carried out between three isolates (631-08^T, 646B-09, 666-09) and between strain 631-08^T and their nearest phylogenetic neighbours, *F. chungangense* CCUG 58910^T, *F. frigidimaris* CCUG 59364^T, *F. hercynium* CCUG 59448^T, *F. aquidurense* CCUG 59847^T and *F. resistens* CCUG 59848^T. DNA was extracted and purified by the method of Marmur [20] and genomic DNA–DNA reassociation analysis was carried out, using hybridization protocols described by Urdiain et al. [28]. Two independent determinations were carried out for each experiment. The results reported are mean values.The G+C content of the DNA of a representative isolate (strain 631-08^T) was determined from the mid-point value (T_m) of the thermal denaturation profile [21] obtained with a Perkin-Elmer UV–Vis Lambda 20 spectrophotometer at 260 nm.

PFGE typing

All 18 trout isolates were characterized by pulsed-field gel electrophoresis (PFGE) profiling of their genomic DNA, after digestion with the restriction enzyme XhoI, according to the specifications of Chen et al. [7], to recognize potential prevalent clones. Similarities between restriction endonuclease digestion profiles were based on visual comparison of the band patterns of isolates run in the same gel by the use of the BioNumerics software (version 3.0; Applied Maths, Kortrijk, Belgium) for comparisons. Strains were considered different when they differed in at least one band [10].

Table 1
Cell fatty acid (CFA) compositions of strains 631-08^T, 646-08, 666-09 and the type strains of related species: 1, *F. onchorhynchi* 631-08^T = CCUG 59446^T; 2, *F. onchorhynchi* 646-08 = CCUG 59310; 3, *F. onchorhynchi* 666-09 = CCUG 59447; 4, *F. chungangense* CCUG 58910^T; 5, *F. hercynium* CCUG 59448^T; 6, *F. aquidurense* CCUG 59847^T; 7, *F. frigidimaris* CCUG 59364^T; 8, *F. resistens* CCUG 59848^T. All strains were cultivated with the same medium and growth conditions. Values are percentages of total fatty acids; fatty acids representing less than 1% in all strains were omitted; –, not detected.

Fatty acid (%)	1	2	3	4	5	6	7	8
$C_{14:0}$	0.7	0.9	0.6	–	1.3	–	1.1	0.3
$C_{14:0}$ aldehyde	0.8	–	0.9	–	1.3	–	–	–
$C_{15:0}$	15.7	23.6	19.0	12.6	10.8	13.2	5.5	5.7
iso-$C_{15:0}$	25.5	24.4	24.3	33.2	31.5	25.1	28.2	41.3
iso-$C_{15:0}$ aldehyde	2.0	0.8	2.1	2.7	2.3	1.3	1.3	–
anteiso-$C_{15:0}$	1.9	1.1	1.9	1.5	1.1	1.5	3.2	1.7
$C_{15:0}$ 3OH	–	1.9	–	–	–	3.9	–	–
iso-$C_{15:0}$ 3OH	5.8	5.7	5.0	8.9	7.8	8.1	7.7	10.1
iso-$C_{15:1}$ G	5.0	3.9	3.5	4.3	3.7	1.8	3.7	3.7
$C_{15:1}$ *ω6c*	7.6	10.9	10.2	8.5	5.3	11.4	4.1	4.5
$C_{16:0}$	2.9	2.9	3.0	1.9	4.4	1.2	2.3	1.4
iso-$C_{16:0}$	1.1	0.7	1.1	–	0.5	0.5	1.0	0.4
$C_{16:0}$ 3OH	2.5	2.4	1.6	–	4.0	0.9	3.5	–
iso-$C_{16:0}$ 3OH	1.5	0.8	1.0	–	–	1.3	1.6	–
iso-$C_{16:1}$ H	0.6	–	0.9	–	–	1.1	1.0	0.4
$C_{16:1}$ *ω7c*	9.8	10.2	10.6	3.5	12.2	6.8	19.2	4.5
iso-$C_{17:0}$ 3OH	5.4	3.7	3.4	7.8	6.4	7.0	5.9	8.7
$C_{17:1}$ *ω8c*	0.9	1.0	1.1	1.1	0.5	0.8	–	–
$C_{17:1}$ *ω6c*	2.4	2.9	2.8	5.9	1.4	5.2	3.5	1.2
iso-$C_{17:1}$ *ω9c*	2.9	2.2	3.1	4.5	2.7	4.9	4.3	5.1
Summed feature 1[a]	1.7	–	1.8	2.2	0.3	–	2.0	2.0
Unidentified fatty acid								
ECL 11.541	1.1	–	1.0	1.6	1.2	0.7	0.8	–
ECL 14.930	–	–	–	–	–	–	–	4.4

[a] Summed feature 1 comprised $C_{17:1}$ ISO I and/or $C_{16:0}$ DMA.

Results and discussion

Comparative 16S rRNA sequence analysis revealed 99.2–100% sequence similarity between the isolates from the diseased trout, demonstrating their high genealogical relatedness. The 16S rRNA gene sequence of strain 631-08^T comparisons with 16S rRNA gene sequences available in GenBank revealed a clear affiliation to the genus *Flavobacterium*, showing the highest sequence similarities with *F. chungangense* (98.6% sequence similarity), *F. frigidimaris* (98.1%), *F. hercynium* (97.9%) and *F. aquidurense* (97.8%). The phylogenetic trees, based on the Neighbour-Joining algorithm (Fig. 1), as well as the other two methods (data not shown), revealed that strain 631-08^T formed a distinct lineage, clustering with the aforementioned species. Although bootstrap resampling analysis did not demonstrate a strong association between strain 631-08^T and these species, the branching position of strain 631-08^T, with respect to its closest phylogenetic relative, *F. chungangense*, was stable, according to the three clustering algorithms used in this study (Fig. 1). Sequence similarities between strain 631-08^T and other *Flavobacterium* species ranged between 90.6–97.5%. The trout isolates were only distantly related of *F. phychrophilum* (93.6% sequence similarity), confirming that they do not belong to this species. This result is consistent with the differences in phenotypic characteristics observed between this species and the trout isolates, as well as the lack of PCR-amplification with *F. psychrophilum* specific-primers [31].

The hybridization analysis between the genomic DNA of isolate 631-08^T and the DNAs of isolates 666-09 and 646B-08 exhibited 82.2 ± 0.2% and 97.3 ± 4.1% similarities, respectively, confirming these strains to be members of the same species [30]. The DNA–DNA hybridization results between strain 631-08^T and its nearest phylogenetic neighbours, *F. chungangense* CCUG 58910^T, *F. frigidimaris* CCUG 59364^T, *F. hercynium* CCUG 59448^T, and *F. aquidurense* CCUG 59847^T were 18.5 ± 7.8%, 26.6 ± 9.8, 23.3 ± 10.6%, 19.5 ± 3.1%, respectively, confirming that the trout isolates constitute a distinct species [30].

The G + C content of 631-08^T was found to be 33.0 mol%; this value was confirmed in three different assays. This DNA G + C content is consistent with what has been observed for species of the genus *Flavobacterium* [4].

The CFA profiles of the isolates from diseased trout were compared with the type strain of the most closely related *Flavobacterium* species, i.e., by 16S rRNA gene sequence comparative analyses (Table 1). The predominant CFAs detected in strains 631-08^T, 646-08 and 666-09 were iso-$C_{15:0}$, $C_{15:0}$ and $C_{16:1}$ *ω7c* (24–26%, 16–24% and 10–11%, respectively). CFAs $C_{15:1}$ *ω6c*, iso-$C_{15:1}$ G, iso-$C_{15:0}$ 3OH and iso-$C_{17:0}$ 3OH were observed in percentages between 3 and 11%. The fatty acid profiles of the isolates from diseased trout are in accordance with those of members of the genus *Flavobacterium* [1,2] and are similar to the profiles of the most closely related species.

Cells of strain 631-08^T contained menaquinone-6 (MK-6) as the major respiratory quinone, in line with all members of the family *Flavobacteriaceae* [1,3]. The polar lipid profile of strain 631-08^T consisted of phosphatidylethanolamine, unknown aminolipids AL1 and AL3, lipids L1, L2, L3 and L4 and phospholipid PL1 (Fig. 2).

Isolates exhibited almost identical phenotypic characteristics, except for the degradation of casein (isolates 947B-08, 950B-08, 45-09, 662-09, 666-09 were negative) and gelatine (isolate 47B-2-09 was positive), reduction of nitrate and nitrite (isolate 47B-2-09 was negative), and production of arginine dihydrolase (isolate 688B-08 was positive). The phenotypic characteristics that differentiate the trout isolates from related phylogenetic species are shown in Table 2. The different response to growth on marine agar, tolerance to 3% NaCl, hydrolysis of gelatine or nitrate reduction clearly differentiated the trout isolates from *F. chungangense* CCUG 58910^T and *F. frigidimaris* CCUG 59364^T, the closet neighbours on the basis of 16S rRNA gene similarity. Isolates of the new species were recovered from diseased rainbow trout in which the presence of *F. psychrophilum* was suspected based on clinical symptoms and epidemiological background. The strains of the new species can be differentiated from this species on the basis of their ability

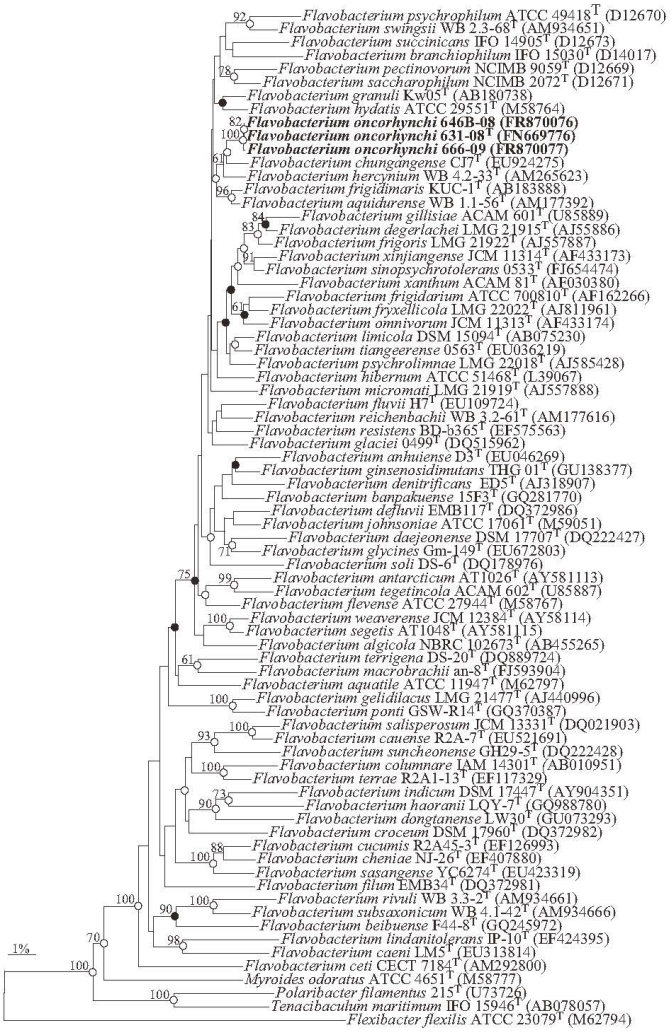

Fig. 1. Partial neighbour-joining phylogenetic tree inferred from comparison of 16S rRNA gene sequences showing the position of strain 631-08T (*Flavobacterium oncorhynchi* sp. nov.) within the genus *Flavobacterium*. Bootstrap values (expressed as a percentage of 1000 replications) equal to or higher than 70% are given at the branching points. Solid circles indicate that the corresponding nodes (groupings) are also obtained on the maximum-likelihood tree. Open circles indicate that the corresponding nodes (groupings) are also obtained on the maximum-likelihood and parsimony trees. *Leeuwenhoekiella marinoflava* ATCC 19326T (M58770) was used as an out-group. Bar, 1% sequence divergence.

to grow on trypticase-soy and nutrient agars, reduce nitrate, β-galactosidase activity and hydrolysis of aesculin and starch, while *F. psychrophilum* isolates give opposite reactions for these characteristics. *F. branchiophilum* and *F. columnare* are also to clinical relevant bacterial fish pathogens that can be differentiated from the new species on the basis of their failure to grow in trypticase-soy and nutrient agars and to hydrolyze aesculine. Two new species, *Flavobacterium chilense* sp. nov., and *Flavobacterium araucanarum* sp. nov., have been recently isolated from farmed diseased rainbow trout and Atlantic salmon in Chile [15]. The isolates of *F. oncorhynchi* can readily be differentiated from both species because they are motile (gliding), grow in 3% NaCl, assimilate mannitol and hydrolyze gelatine, while *F. oncorhynchi* gives opposite results. In addition, this species can also be differentiated from *F. chilense* by the ability of the latter species to hydrolyze casein, produce acid from L-arabinose and assimilate gluconate and malate. Moreover,

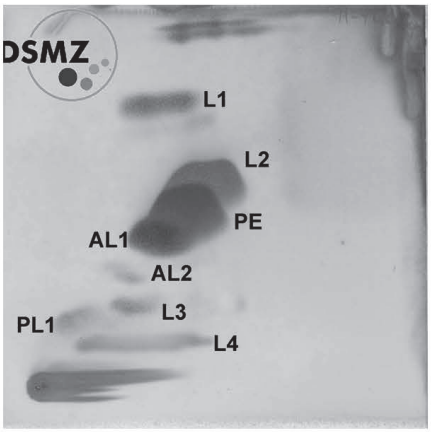

Fig. 2. Two-dimensional TLC of polar lipids of strain 631-08^T. *Abbreviations*: PE, phosphatidylethanolamine; AL, unidentified aminolipid; L1, L2, L3, L4, unidentified polar lipids; PL1, unidentified phospholipid.

differences were also observed in the composition of polar lipids. Phosphatidylserine was one of the major polar lipids observed in *F. chilense* and *F. araucananum* but it was not detected from the extract from strain 631-08^T of *F. oncorhynchi*.

Visual comparison of the restriction endonuclease digestion profiles generated by PFGE, revealed 7 different profiles (represented by isolates 441B-08, 688B-08, 666-09, 631-08^T, 646-08, 47B-2-09 and 425B-08) among the 18 trout isolates (Fig. 3). Most isolates were closely related and exhibited genetic similarities ≥80% supporting that they are members of a single species. Some isolates with identical PFGE profile were repeatedly isolated from different diseased trout on different times, data that could be indicative of their clinical significance. Nevertheless the virulence of the new isolates has not been determined yet by challenge experiments.

In conclusion, it is clear from the results of this study conducted using a polyphasic taxonomic approach, that the unidentified isolates from rainbow trout constitute a new species of the genus *Flavobacterium*, for which the name *Flavobacterium oncorhynchi* sp. nov. is proposed.

Table 2
Characteristics that differentiate *Flavobacterium oncorhynchi* sp. nov. from related *Flavobacterium* species. Strains: 1, *F. oncorhynchi* 631-08^T; 2, *F. chungangense* CCUG 58910^T; 3, *F. frigidimaris* CCUG 59364^T; 4, *F. hercynium* CCUG 59448^T; 5, *F. aquidurense* CCUG 59847^T. Data are taken from this study. All taxa have MK-6 as major respiratory quinone. +, positive reaction; –, negative reaction.

Characteristic	1	2	3	4	5
Growth on:					
Trypticase-soy agar	+	+	+	–	+
Marine agar	–	+	–	–	–
Growth at 30 °C	+	+	–	–	+
Tolerance to 3% NaCl	–	+	+	–	–
Gliding motility	–	–	+	+	–
Flexirubin-type pigment	+	–	+	+	–
Acid production from carbohydrates	–	–	+	–	–
Assimilation of:					
Arabinose	+	+	+	–	+
Mannitol	–	–	+	+	–
N-acetyl glucosamine	+	–	+	+	+
Citrate	–	–	–	+	–
Hydrolysis of:					
Gelatin	–	+	+	+	–
Casein	+	–	+	+	+
L-Tyrosin	+	–	+	+	–
β-Galactosidase activity	+	–	+	–	+
Nitrate reduction	+	–	–	+	–
G–C content (mol%)	33.0	34.5	37.5	34.5	33.5

Description of Flavobacterium oncorhynchi sp. nov.

Flavobacterium oncorhynchi (on.co.rhyn'chi. N.L. gen. n. oncorhynchi of Oncorhynchus, named after the rainbow trout, *Oncorhynchus mykiss*, from which the type strain was isolated).

Cells are Gram-negative rods, 0.5 μm wide and 2–3 μm long, nonspore-forming, and non-gliding. Grows well under aerobic conditions, and grows weakly under microaerobic conditions, respectively. Grows at 15–30 °C, with optimal growth at 25 °C. Cells do no growth at 37 °C and 42 °C. Growth occurs on trypticase-soy and nutrient agars but not on MacConkey and marine agars after incubation at 25 °C for 72 hours. Growth does not occur in brain heart infusion broth containing 3%, 4.5 and 6.5% NaCl. Colonies are circular, pale yellow-pigmented, smooth, and entire on TGE agar after 72 h of incubation at 25 °C. Colonies are non-haemolytic on sheep blood agar. Diffusible flexirubin-type pigments are produced. Congo red is not absorbed by colonies. Catalase and oxidase are produced. Nitrate and nitrite are reduced. Starch is degraded but gelatin and agar are not. Degradation of casein, was variable (631-08^T was positive). A brown pigment is produced on tyrosine agar although degradation of tyrosine was not detected. Aesculin is hydrolysed but not arginine, DNA and urea. Indole and H_2S

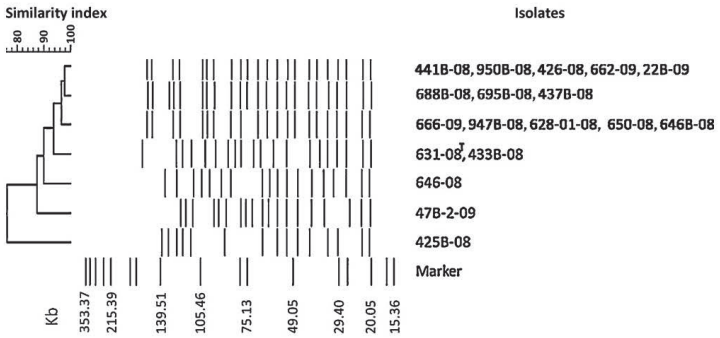

Fig. 3. Dendrogram showing a schematic representation of the different XhoI PFGE macrorestriction patters exhibited by *F. oncorhynchi* isolates.

are not produced. Activity for β-galactosidase is detected. Acid is not produced from D-glucose. Arabinose, mannose, N-acetyl-glucosamine, maltose are used as sole carbon and energy source but not mannitol, gluconate, caprate, adipate, citrate and malate. Cells contain menaquinone-6 (MK-6) as the major respiratory quinone. The major cellular fatty acids are iso-$C_{15:0}$, $C_{15:0}$ and $C_{16:1}$ $\omega 7c$. The polar lipid profile of strain 631-08^T contained phosphatidylethanolamine, unknown aminolipids AL1 and AL3, lipids L1, L2, L3 and L4 and phospholipid PL1.

The type strain, 631-08^T (=CECT 7678^T = CCUG 59446^T), was isolated from the liver of a rainbow trout. The DNA G + C content of this strain is 33.0 mol%.

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Flavobacterium plurextorum sp. nov. Isolated from Farmed Rainbow Trout (*Oncorhynchus mykiss*)

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Abstract

Five strains (1126-1H-08^T, 51B-09, 986-08, 1084B-08 and 424-08) were isolated from diseased rainbow trout. Cells were Gram-negative rods, 0.7 μm wide and 3 μm long, non-endospore-forming, catalase and oxidase positive. Colonies were circular, yellow-pigmented, smooth and entire on TGE agar after 72 hours incubation at 25°C. They grew in a temperature range between 15°C to 30°C, but they did not grow at 37°Cor 42°C. Based on 16S rRNA gene sequence analysis, the isolates belonged to the genus *Flavobacterium*. Strain 1126-1H-08^T exhibited the highest levels of similarity with *Flavobacterium oncorhynchi* CECT 7678^T and *Flavobacterium pectinovorum* DSM 6368^T (98.5% and 97.9% sequence similarity, respectively). DNA–DNA hybridization values were 87 to 99% among the five isolates and ranged from 21 to 48% between strain 1126-1H-08^T, selected as a representative isolate, and the type strains of *Flavobacterium oncorhynchi* CECT 7678^T and other phylogenetic related *Flavobacterium* species. The DNA G+C content of strain 1126-1H-08^T was 33.2 mol%. The predominant respiratory quinone was MK-6 and the major fatty acids were iso-$C_{15:0}$ and $C_{15:0}$. These data were similar to those reported for *Flavobacterium* species. Several physiological and biochemical tests differentiated the novel bacterial strains from related *Flavobacterium* species. Phylogenetic, genetic and phenotypic data indicate that these strains represent a new species of the genus *Flavobacterium*, for which the name *Flavobacterium plurextorum* sp. nov. was proposed. The type strain is 1126-1H-08^T (=CECT 7844^T=CCUG 60112^T).

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Competing Interests: One of the authors, Dr. M.A.Palacios is affiliated to Piszolla, S.L., 37800 Alba de Tormes (Salamanca), Spain. She is the technical manager of the company that identified infectious problems in one of their fish farms and she has collaborated with us for describing the new *Flavobacterium* species. So, this does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

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Introduction

The genus *Flavobacterium* is the type genus of the family *Flavobacteriaceae* accommodating Gram-negative, non-endospore-forming, aerobic, oxidase-positive, non-fermenting, predominantly gliding, yellow-pigmented bacteria [1,2]. The genus, initially described to accommodate seven species, has considerably expanded with the description of many new species. Currently it includes 99 species, many of them described during the last five years. [3]. Members of the genus *Flavobacterium* can been isolated from a number of diverse habitats such as soil, water, sludge, plants, food products such as fish, meat, poultry, milk or lactic acid beverages [2,4]. Most species are non-pathogenic, although a number of species have been associated with different clinical infections, being freshwater fish the animals most prone to flavobacterial infections [5]. Some *Flavobacterium* species, mainly *Flavobacterium columnare*, *Flavobacterium branchiophilum* and *Flavobacterium psychrophilum*, are well-recognized fish pathogens responsible for important economic losses in the fish farming industry [6,7]. However, several other species such as *Flavobacterium hydatis*, *Flavobacterium jhonsoniae*, *Flavobacterium succinicans*, *Flavobacterium chilense*, *Flavobacterium araucananum* or *Flavobacterium oncorhynchi* have been also associated with infections in fish [1,4,5,8–10]. Additionally, a number of new *Flavobacterium* species also have been described from the water of aquaculture facilities [11–13]. This plethora of *Flavobacterium* species could reproduce the diversity of flavobacteria associated with fish or fish surrounding environments. Some of these species could be considered commensal and opportunistic pathogenic bacteria [4], which point out the necessity for an accurate identifications of those strains of *Flavobacterium* spp. isolated from fish or fish farm environments. However, such identifications are extremely difficult based exclusively on biochemical criteria [4,8,14] and must be complemented with chemotaxomic and genetic methods [4,5].

In this article, we report the phenotypic, genotypic and phylogenetic characterization of five novel *Flavobacterium*-like strains isolated from diseased trout. Based on the presented findings, a new species of the genus *Flavobacterium*, *Flavobacterium plurextorum* sp. nov., is proposed.

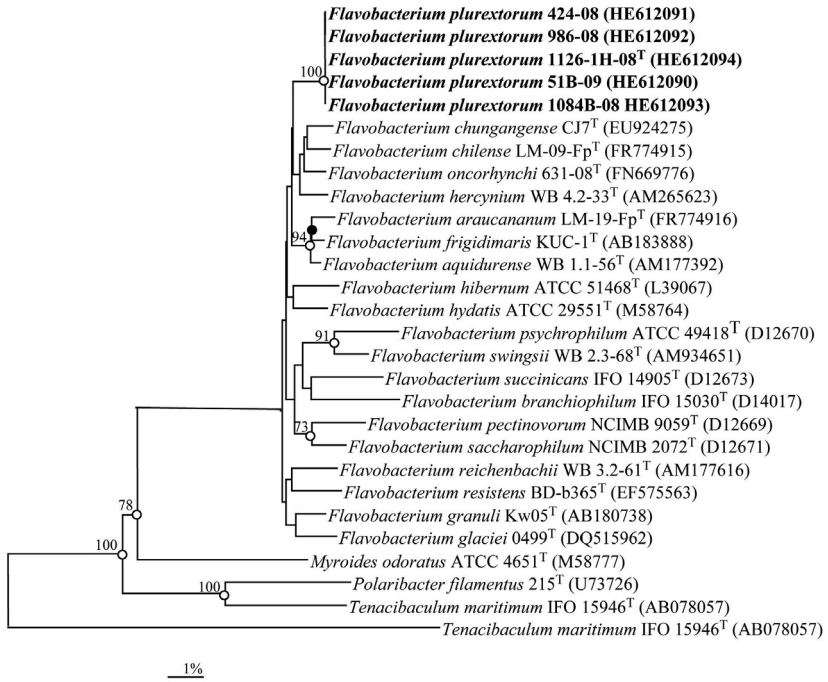

Figure 1. Phylogenetic tree based on 16S rRNA gene sequence comparisons, obtained with the neighbour-joining algorithm, showing the relationships of *Flavobacterium plurextorum* sp. nov. with related species. *Flexibacter flexilis* ATCC 23079^T was used as an outgroup. Bootstrap values (expressed as a percentage of 1,000 replications) greater than 70% are given at the nodes. Solid circles indicate that the corresponding nodes (groupings) are also obtained on the maximum-likelihood tree. Open circles indicate that the corresponding nodes (groupings) are also obtained on the maximum-likelihood and parsimony trees. Sequence accession numbers are indicated in brackets. Bar, 1% sequence divergence.
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Materials and Methods

The present work does not include any experimental infections trial with farmed trout, just trout exclusively were used to identify microbiologically the etiological agent of the bacterial septicemia. Therefore, we did not consult with the IACUC and no specific national regulations for these procedures are available. Nevertheless, in order to ensure the welfare and ameliorate suffering of trout during transportation to the laboratory and euthanasia, trout were handled according to guidelines of relevant international organisms such as OIE (http://www.oie.int/doc/ged/D7821.PDF) and AVMA (https://www.avma.org/KB/Policies/Documents/euthanasia.pdf) and they were further necropsied under aseptic conditions. In addition, these procedures were approved by the responsible of animal welfare of the UCM Animal Health Department. The trout were sacrificed for the purpose of the study and the sacrifice was approved by the Technical Manager (Mari Angel Palacios, DVM, PhD) of the fish farm located in the west of Spain.

Trout and Strain Isolation

A clinical episode of septicemia occurred in a rainbow trout (*Oncorhynchus mykiss*) farm located in the central region of Spain. Affected trout were submitted by the Technical Manager of the fish farm to the Animal Health Surveillance Centre (VISAVET) of the Universidad Complutense (Madrid, Spain) for a confirmatory microbiological diagnosis.

Five Gram-negative, rod-shaped bacteria were isolated from liver (strains 986-08 and 424-08), gills (strains 1084B-08 and 51B-09) and eggs (1126-1H-08^T) of five different trout. The strains were recovered in two different years (2008 and 2009) and they were isolated on tryptone glucose extract agar (TGE; Difco) after incubation at 25°C for 72 hours under aerobic conditions.

Phylogenetic Analysis

A large continuous sequence (approximately 1,400 bases) of the 16S rRNA gene of five strains was determined bidirectionally using universal primers pA (5′- AGAGTTTGATCCTGGCTCAG, positions 8–27, *Escherichia coli* numbering) and pH* (5′-AAGGAGGTGATCCAGCCGCA, positions 1541–1522, *E. coli* numbering) as described previously [10], and subjected to a comparative analysis. The identification of the phylogenetic relatives and calculations of pair-wise 16S rRNA gene sequence similarities were achieved, using the EzTaxon-e server [15]. The 16S rRNA gene sequences of the type strains of all validly published species of the genus *Flavobacterium* were retrieved from

Table 1. Cellular fatty acid compositions of *Flavobacterium plurextorum* 1126-1H-08^T and its closest phylogenetic neighbours.

Fatty acid	1	2	3	4	5	6	7	8
Saturated								
$C_{12:1}$	1	tr	–	–	–	–	tr	–
$C_{14:0}$	tr	–	tr	1.1	tr	tr	–	tr
$C_{15:0}$	15	13.5	11.9	5.5	5.6	6.9	20.6	15.7
$C_{16:0}$	2	1.6	1.1	2.3	2.8	2.2	tr	2.9
Hydroxy								
$C_{15:0}$ 2OH	–	–	1.1	–	tr	–	–	–
$C_{15:0}$ 3OH	3	3.3	1.9	–	–	–	1.8	–
iso-$C_{15:0}$ 3OH	6	7.8	6.9	7.7	8.6	5.8	7.1	5.8
$C_{16:0}$ 3OH	3	–	1.1	3.5	4.5	1.4	–	2.5
iso-$C_{16:0}$ 3OH	2	1.0	2.1	1.6	1.9	tr	2.1	1.5
iso-$C_{17:0}$ 3OH	5	8.2	7.3	5.9	10.3	5.1	7.0	
Branched								
$C_{14:0}$ aldehyde	1.0	–	–	–	–	–	–	tr
iso-$C_{15:0}$	19	26.1	14.6	28.2	23.5	28.0	24.8	25.5
anteiso-$C_{15:0}$	1.0	1.3	3.0	3.2	tr	4.3	2.5	1.9
iso-$C_{15:0}$ aldehyde	2.0	3.2	1.2	1.3	tr	1.3	2.3	2.0
iso-$C_{15:1}$ G	6.0	2.9	7.4	3.7	5.8	7.2	5.0	5.0
iso-$C_{16:0}$	1	–	1.1	1.0	tr	1.0	–	1.1
iso-$C_{16:1}$ H	tr	–	1.0	1.0	tr	–	–	tr
iso-$C_{17:1}$ ω9c	3	6.0	5.2	4.3	4.1	6.0	1.1	2.9
Unsaturated								
$C_{15:1}$ ω6c	9	12.3	10.1	4.1	2.9	5.5	12.2	7.6
$C_{16:1}$ ω7c	10	3.7	11.2	19.2	15.7	18.1	2.2	9.8
$C_{17:1}$ ω6c	6	5.9	6.4	3.5	2.5	3.2	6.2	2.4
$C_{17:1}$ ω8c	1	1.0	1.5	–	tr	tr	1.3	tr
Summed feature 1[a]	–	–	–	2.0	1.7	1.4	–	1.7
Unidentified fatty acid[b]								
ECL 11.541	2	1.4	tr	tr	tr	tr	1.2	1.1
ECL 12.555	1	–	tr	–	–	–	1.1	tr
ECL 14.809	1	–	–	–	–	–	–	–
ECL 16.580	–	–	tr	–	1.1	–	–	tr

Taxa: 1, *F. plurextorum* 1126-1H-08^T; 2, *F. pectinovorum* CCUG 58916^T; 3, *F. aquidurense* CCUG 59847^T; 4, *F. frigidimaris* CCUG 59364^T; 5, *F. hydatis* DSM 2063^T; 6, *F. araucananum* CCUG 61031^T; 7, *F. chungangense* CCUG 58910^T; 8, *F. oncorhynchi* CECT 7678^T.
Values are percentages of total fatty acids; fatty acids representing less than 1% in all strains were omitted. tr = trace amount, i.e., <1%. - = not detected.
CFA values for type strains other than *F. plurextorum* 1126-1H-08^T were taken from the CCUG culture collection (http://www.ccug.se/). Strains were cultivated on the same medium and growth conditions.
[a]Summed features represent groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 1 comprised iso-$C_{17:1}$ I/$C_{16:0}$ DMA.
[b]ECL, equivalent chain length.
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GenBank and aligned with the newly determined sequences using the program SeqTools [16]. Phylogenetic trees were constructed according to three different algorithms: neighbour-joining [17], using the programs SeqTools and TREEVIEW [18]; maximum-likelihood, using the PHYML software [19]; and maximum-parsimony, using the software package MEGA (Molecular Evolutionary Genetics Analysis) version 5.0 [20]. Genetic distances for the neighbour-joining and the maximum-likelihood algorithms were calculated by the Kimura two-parameter [21] and close-neighbour-interchange (search level = 2, random additions = 100) was applied in the maximum-parsimony analysis. The stability of the groupings was estimated by bootstrap analysis (1000 replications).

Genomic DNA G+C Content Determination and DNA-DNA Hybridizations

The G+C content of the genomic DNA of a representative strain (1126-1H-08^T) was determined from the mid-point value (Tm) of the thermal denaturation profile [22], obtained with a Perkin-Elmer UV-Vis Lambda 20 spectrophotometer at 260 nm.

Genomic DNA-DNA hybridizations were carried out between strains 1126-1H-08^T, 986-08, 424-08, 1084B-08 and 51B-09, and between strain 1126-1H-08^T and the type strains of the closest

Table 2. Characteristics that differentiate *Flavobacterium plurextorum* sp. nov. from closely related *Flavobacterium* species based in the 16S rRNA tree topology.

Characteristic	1	2	3	4	5	6	7	8
Growth on Marine agar	−	−	−	+	−	−	−	−
Growth at 30°C	+	+	+	−	+	+	+	+
Hydrolysis of:								
L- tyrosine	+	−	+	−	+	−	−	+
DNA	−	−	−	−	+	−	+	−
Urea	−	−	+	−	−	−	−	−
Nitrate reduction	+	+	−	−	+	+	+	+
Assimilation of:								
Arabinose	+	+	+	+	−	+	+	+
Mannitol	−	−	−	+	−	−	−	−
N-acetyl-glucosamine	+	+	+	+	+	+	-	+
Production of:								
Valine arylamidase	−	+	+	+	+	+	+	−
α-Glucosidase	+	−	−	+	+	+	−	+
β-Glucosidase	−	+	−	+	−	+	+	−
N-Acetyl-β-glucosaminidase	+	−	−	+	+	−	−	+

Taxa: 1, *F. plurextorum* 1126-1H-08^T; 2, *F. pectinovorum* CCUG 58916^T; 3, *F. aquidurense* CCUG 59847^T; 4, *F. frigidimaris* CCUG 59364^T; 5, *F. hydatis* DSM 2063^T; 6, *F. araucananum* CCUG 61031^T; 7, *F. chungangense* CCUG 58910^T; 8, *F. oncorhynchi* CECT 7678^T.
Data are from this study.
+, positive reaction; −, negative reaction.
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phylogenetically related species. DNA was extracted and purified by the method of Marmur [22]. Hybridization studies were carried out, using the membrane method of Johnson [23], described in detail by Arahal *et al.* [24]. The hybridization experiments were carried out under optimal conditions, at a temperature of 44°C, which is within the limits of validity for the membrane method [25]. The percentages of hybridization were calculated as described by Johnson [26]. Three independent determinations were carried out for each experiment and the results reported as mean values. The type strains of species *F. aquidurense* CCUG 59847^T, *F. araucananum* CCUG 61031^T, *F. hydatis* DSM 2063^T, *F. pectinovorum* CCUG 58916^T, *F. frigidimaris* CCUG 59364^T, *F. chungangense* CCUG 58910T and *F. oncorhynchi* CECT 7678^T were included in this study.

Chemotaxonomic Characteristics

Respiratory quinones of strain 1126-1H-08^T were extracted from 100 mg of freeze-dried cell material, using the two stage method described by Tindall [27,28], and further separated by thin layer chromatography on silica gel and analyzed, using HPLC, by the identification service of the DSMZ (Braunschweig, Germany).

For cell fatty acid-fatty acid methyl ester (CFA-FAME) analyses, strain 1126-1H-08^T was grown on Columbia II agar base (BBL 4397596) with 5% horse blood, at 30°C for 30–48 h, under aerobic conditions. The CFA-FAME profile was determined using gas chromatography (Hewlett Packard HP 5890) and a standardized protocol similar to that of the MIDI Sherlock MIS system [29], described previously [10]. CFAs were identified and the relative amounts were expressed as percentages of the total fatty acids of the respective strains.

Morphological, Physiological and Biochemical Characteristics

The minimal standards for the description of new taxa in the family *Flavobacteriaceae* [30] were followed for the phenotypic characterization of the strains. Gram-staining was performed as described by Smibert & Krieg [31]. Oxidase activity was determined by monitoring the oxidation of tetramethyl-*p*-phenylenediamine on filter paper and catalase activity was determined, using 3% H_2O_2 solution [31]. Hydrolysis of L-tyrosine (0.5%, w/v), lecithin (5%, w/v) [31], esculin (0.01% esculin and 0.05% ferric citrate, w/v), gelatin (4%; w/v), starch (0.2%, w/v), and casein [50% skimmed milk (Difco), v/v] were tested using nutrient agar as basal medium [30]. DNase test agar (Difco) was used for the DNase assay. Hydrolysis of urea (1%, w/v) was tested as described by Bowman *et al.* [32]. Growth in brain heart infusion broth was assessed at 15, 25, 30, 37 and 42°C, with 3.0, 4.5 and 6.5% added NaCl, and under anaerobic (with 4–10% CO_2) and micro-aerobic (with 5–15% O_2 and 5–12% CO_2) conditions, using GasPak Plus and CampyPak Plus systems (BBL), respectively. Growth was tested on MacConkey (bioMérieux), nutrient (Difco) and trypticase-soy (bioMérieux) agar plates. The presence of gliding motility, using the hanging drop technique, and the production of flexirubin-type pigments and extracellular glycans were assessed, using the KOH and Congo red tests, respectively [1]. The strains were further biochemically characterized using the API 20NE and API ZYM systems (bioMérieux) according to the manufacturer's instructions, except that incubation temperature was 25°C. The type strains of species *F. aquidurense* CCUG 59847^T, *F. araucananum* CCUG 61031^T, *F. hydatis* DSM 2063^T, *F. pectinovorum* CCUG 58916^T, *F. frigidimaris* CCUG 59364^T, *F. chungangense* CCUG 58910^T and *F. oncorhynchi* CECT 7678^T were included in this study as references for the investigation of the phenotypic properties of the trout strains, using the same laboratory conditions.

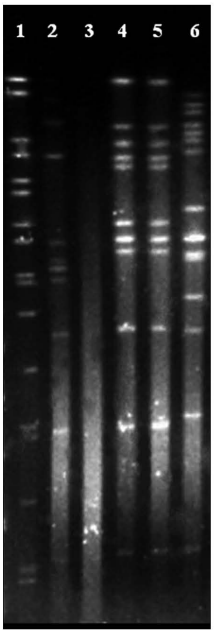

Figure 2. PFGE patterns generated after Bsp120I macrorestriction of *Flavobacterium plurextorum* sp. nov. Lane 1, DNA molecular size marker; Lanes 2 to 6, strains 1126-1H-08^T, 51B-09, 986-08, 1084B-08 and 424-08, respectively.
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PGFE Typing

The five strains were characterized by pulsed-field gel electrophoresis (PFGE), after digestion of their genomic DNAs with the restriction enzymes *Bsp120*I and *Xho*I, according to the specifications of Chen *et al.* [33]. DNA fragments were resolved in a 1% agarose gel with a pulse-field gel electrophoresis apparatus, CHEF-DR III (Bio-Rad), at 6V/cm for 40 hours, with switching times ramped from 0.1 to 12 s at 14°C, with an angle of 120°. The gels were stained for 30 min with Syber-Safe and photographed under UV light (Gel-Doc, Bio-Rad). Strains differing in at least one band were considered different.

Results and Discussion

16S rRNA gene sequences were determined for the five trout strains, displaying 100% 16S rRNA sequence similarity among them. Sequence searches showed that the 16S rRNA gene sequence of the strains were most similar to those of species of the genus *Flavobacterium*, exhibiting the highest levels of similarity with the sequence of the type strains of *Flavobacterium oncorhynchi* CECT 7678^T and *Flavobacterium pectinovorum* DSM 6368^T (98.5% and 97.9% sequence similarity, respectively). In addition, strains exhibited 16S rRNA gene sequence similarities greater than 97.0% with other seventeen other *Flavobacterium* species. It is clear from the phylogenetic analysis (Fig. 1) that the trout strains held a clear affiliation to the genus *Flavobacterium* and represented a distinct sub-lineage clustering with a cluster of four species that included *F. pectinovorum*, *F. chilense*, *F. oncorhynchi* and *F. hercynium*. However, their position within this sub-group was not supported by significant bootstrap values. The GenBank accession numbers for the 16S rRNA gene sequences of five strains sequenced in this study are shown in Fig. 1.

Genomic DNA–DNA hybridizations between the trout strains yielded binding values of 87 to 100%. *Flavobacterium* species with 16S rRNA gene sequence similarities to the sequences of the trout strains lower than 98.0% correlated with levels of genomic DNA-DNA relatedness always lower than 70% [9–11,34–36]. For that reason, DNA-DNA hybridizations were carried out only between strain 1126-1H-08^T and the type strains of the phylogenetically closest related species; *i.e.*, those species with 16S rRNA gene sequence similarities greater than 97.5%. The levels of DNA-DNA relatedness for strain 1126-1H-08^T with respect to *F. aquidurense* CCUG 59847^T, *F. araucananum* CCUG 61031^T, *F. hydatis* DSM 2063^T, *F. pectinovorum* CCUG 58916^T, *F. frigidimaris* CCUG 59364^T, *F. chungangense* CCUG 58910^T and *F. oncorhynchi* CECT 7678^T ranged between 21 and 48%. These values were below the 70% cut-off point for species delineation [37,38] and clearly confirmed that the trout strains belong to a distinct genomic species of the genus *Flavobacterium*. The DNA G+C content of strain 1126-1H-08^T was 33.2 mol%, a value consistent with those of the genus *Flavobacterium* [1,30].

Chemotaxonomic characteristics of strain 1126-1H-08^T were in accordance with those of members of the genus *Flavobacterium* [5,6]: the major quinone was MK-6 (95%) with minor amounts of MK-5 (5%). The predominant cell fatty acids of strain 1126-1H-08^T were iso-$C_{15:0}$ (19%) and $C_{15:0}$ (15%). Strain 1126-1H-08^T also contained moderate or small amounts of $C_{16:1}$ *ω7c* (10%), $C_{15:1}$ *ω6c* (9%), iso-$C_{15:0}$ 3-OH, $C_{17:1}$ *ω6c*, isoG-$C_{15:1}$ (6%/each), iso-$C_{17:0}$ 3-OH (5%), iso-$C_{17:1}$ *ω9c*, $C_{15:0}$ 3-OH, $C_{16:0}$ 3-OH (3%/each), isoaldehyde-$C_{15:0}$, $C_{16:0}$, iso-$C_{16:0}$ 3-OH, unknown fatty acids with an equivalent chain length of 11.5 (2%/each) and $C_{17:1}$ *ω8c*, iso-C16:0, $C_{12:1}$, aldehyde-$C_{14:0}$, anteiso-$C_{15:0}$ and unknown fatty acids with an equivalent chain lengths of 14.8 and 12.5 (1%/each) (Table 1).

The trout strains exhibited identical physiological and biochemical characteristics. Cells were Gram-negative rods, 0.7 µm wide and 3 µm long, non-endospore-forming, and non-gliding. Strains grew well under aerobic conditions and grew weakly under micro-aerobic conditions. Strains grew at 15–30°C with optimal growth at approximately 25°C, while no growth was observed at 37°C or 42°C. Growth occurred on trypticase-soy and nutrient agars but not on Marine agar after incubation at 25°C for 72 hours. Colonies were circular, yellow-pigmented, smooth and entire on TGE agar after 72 hours incubation at 25°C. Colonies are non-hemolytic on Columbia agar after 72 hours incubation at 25°C. Diffusible flexirubin-type pigments were produced and congo red was not absorbed by colonies. Growth did not occur in brain heart infusion broth containing 3, 4.5 and 6.5% NaCl. Catalase and oxidase were produced and nitrate and nitrite were reduced. Starch and tyrosine were degraded but DNA, gelatin, casein or agarose were not. A brown pigment was not produced on tyrosine agar. Aesculin was hydrolyzed but not urea, lecithin and arginine. Indole and H_2S were not produced. Acid was not produced from D-glucose. Arabinose, D-glucose, mannose, N-acetyl-glucosamine, and maltose were used as sole carbon and energy sources but not citrate, mannitol, gluconate, caprate, adipate, and malate. Activities for alkaline phosphatase, leucine arylamidase, N-acetyl-β-glucosaminidase, α-glucosidase, acid phosphatase, and naphthol-AS-BI-phophohydrolase were detected. Esterase C4, valine arylamidase, β-galactosidase, ester lipase C8, lipase C14, cystine arylamidase, α-chymotrypsin, trypsin, α-

galactosidase, β-glucuronidase, β-glucosidase, α-mannosidase and α-fucosidase were not detected.

The phenotypic characteristics that differentiated the trout strains from phylogenetically related species are shown in Table 2. The new species also can be also differentiated from the clinically relevant fish pathogens *F. columnare*, *F. psycrophilum* and *F. branchiophilum*, by the inability of these three species to grow in trypticase-soy agar and to hydrolyze aesculin [4]. Other species isolated from diseased fish such as *F. hydatis*, *F. jonshomiae* and *F. succinicans* are motile (gliding), degrade DNA and produce acid from carbohydrates [4], while the new species exhibited opposite results for those tests. Moreover, the new species can be readily differentiated from *F. chilense* and *F. araucananum* because the latter species are motile (gliding), grow in 3% NaCl and assimilate mannitol [9] and from *F. oncorhynchi* which produces β-galactosidase while the new species give opposite results for this test [10].

After PFGE typing, the trout strains were characterized by 3 different restriction profiles with the enzymes *Bsp120*I (Fig. 2) and *Xho*I (not shown). Strains 986-08 and 1084B-08 exhibited indistinguishable restriction profiles with both enzymes and strain 51B-09 could not be characterized because its DNA systematically was autodegraded.

Flavobacteria are known to belong to the microbiota of fish and fish eggs [4,5]. Therefore, although two strains were isolated from internal organs, the other three were recovered from gills and eggs which suggest that the new species could be saprophytic or commensal and able to colonize fish, and produce disease under stressful conditions or other predisposing circumstances such as coinfections with other bacteria or viruses, poor farming conditions or environmental disorders [4,39]. This assumption should be confirmed by experimental infection trials. Nevertheless, the formal description of *Flavobacterium plurextorum* and the availability of tests to facilitate its identification from other *Flavobacterium* species associated with fish disease or isolated from diseased fish will aid laboratories in its recognition and identification in the future, and to improve the knowledge of its distribution and possible association with disease.

Conclusion

The phylogenetic, genotypic and phenotypic results of the present polyphasic study demonstrated that the new strains isolated from rainbow trout represented a novel species of the genus *Flavobacterium*, for which the name *Flavobacterium plurextorum* sp. nov. is proposed (plu.rex.to'rum. L. comp. pl. plures, more, several, many; L. pl. n. exta -orum, entrails; N.L. gen. pl. n. plurextorum, of several internal organs). Detailed description of the morphological, physiological and biochemical characteristics of this species were indicated above. The type strain is 1126-1H-08^T (=CECT 7844^T=CCUG 60112^T).

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Author Contributions

Conceived and designed the experiments: JFF-G AIV LD. Performed the experiments: LZ CS-P. Analyzed the data: ERBM AV AIV. Wrote the paper: LZ JFF-G AIV. Obtained clinical specimens: MAP. Critical revision and final approval: ERBM AV JFF-G.

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Flavobacterium tructae sp. nov. and *Flavobacterium piscis* sp. nov., isolated from farmed rainbow trout (*Oncorhynchus mykiss*)

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Four Gram-staining-negative, catalase- and oxidase-positive, pale-orange pigmented bacterial strains (435-08^T, 47B-3-09, 412R-09^T and 60B-3-09) were isolated from diseased rainbow trout. Analysis of their 16S rRNA gene sequences suggested their adscription to the genus *Flavobacterium*. Strains formed two phylogenetic groups represented by strains 435-08^T and 47B-3-09 (group A), and strains 412R-09^T and 60B-3-09 (group B) displaying 16S rRNA sequence similarities greater than 99.8–99.9 % within their respective groups. Strain 435-08^T exhibited the highest levels of similarity with *Flavobacterium aquidurense* WB-1.1.56^T (98.6 % sequence similarity) and strain 412R-09^T with *Flavobacterium frigidimaris* KUC-1^T and *Flavobacterium aquidurense* WB-1.1.56^T (98.9 % and 98.6 % sequence similarity, respectively). DNA–DNA hybridization studies showed low levels of relatedness between strain 435-08^T and strain 412R-09^T and between both strains and the most closely related species of the genus *Flavobacterium*. The genomic DNA G+C contents of strains 435-08^T and 412R-09^T were 36.2 and 34.3 mol%, respectively. The predominant respiratory quinone of both strains was MK-6 and the major fatty acids were iso-$C_{15:0}$, $C_{16:1}\omega7c$ and $C_{15:0}$. The two groups of strains could be distinguished from each other and from related species of the genus *Flavobacterium* by a number of phenotypic properties. Phylogenetic, genotypic and phenotypic evidence indicated that strains of groups A and B represent two novel species of the genus *Flavobacterium*, for which the names *Flavobacterium tructae* sp. nov. (type strain 435-08^T=CECT 7791^T=CCUG 60100^T) and *Flavobacterium piscis* sp. nov. (type strain 412R-09^T=CECT 7911^T=CCUG 60099^T) are proposed.

The genus *Flavobacterium* is a member of the family *Flavobacteriaceae*, phylum *Bacteroidetes*, and represents one of the genera with a rapidly increasing number of species. At the time of writing, the genus *Flavobacterium* comprised 90 described species with validly published names (Euzéby, 1997; http://www.bacterio.net). Members of this genus have been isolated from a variety of environmental sources, including soil, water, sludge, plants, food products such as fish, meat, poultry, milk and lactic acid beverages, and human clinical specimens (Bernardet & Nakagawa, 2006; Bernardet & Bowman, 2006). Several species of the genus *Flavobacterium* have been isolated from the fish farming environment, and some of them are (or potentially are) the aetiological agents of fish diseases, including *Flavobacterium*

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 435-08^T, 412R-09^T, 60B-3-09 and 47B-3-09 are HE612100, HE612101, HE774303 and HE774301, respectively.

Two supplementary figures are available with the online version of this paper.

columnare, *F. branchiophilum*, *F. hydatis*, F. johnsoniae, *F. psychrophilum* and *F. succinicans* (Bernardet *et al.*, 1996; Crump *et al.*, 2001; Darwish *et al.*, 2004; Figueiredo *et al.*, 2005; Bernardet & Bowman, 2006; Flemming *et al.*, 2007). Furthermore, three novel species isolated from diseased fish have been described recently, *Flavobacterium chilense*, *Flavobacterium araucananum* and Flavobacterium oncorhynchi (Kämpfer *et al.*, 2012; Zamora *et al.*, 2012). In this article, we report the phenotypic, genotypic and phylogenetic characterization of four novel *Flavobacterium*-like strains isolated from diseased trout. Based on the presented findings, two novel species of the genus *Flavobacterium*, *Flavobacterium tructae* sp. nov. and *Flavobacterium piscis* sp. nov., are proposed.

During the routine microbiological diagnosis from different clinical specimens of rainbow trout (*Oncorhynchus mykiss*) submitted to the Animal Health Surveillance Centre (VISAVET) of the Universidad Complutense (Madrid, Spain), four novel Gram-staining-negative, rod-shaped bacteria were recovered from the liver (strain 435-08^T), kidney (strain 412R-09^T) and gills (strains 47B-3-09 and 60B-3-09) of four different trout with presumptive diagnoses of septicaemia. The fish and strains were recovered from three different ponds (435-08^T from one pond, 412R-09^T from another pond and 47B-3-09 and 60B-3-09 from a third pond) in the same fish farm, in two different years (2008 and 2009). The strains were isolated on tryptone glucose extract agar (TGE; Difco) after incubation at 25 °C for 72 h under aerobic conditions.

In order to establish the phylogenetic allocation of the bacteria, the sequence of a large continuous fragment (approx. 1400 bp) of the 16S rRNA gene of the four strains was obtained in both directions, using the universal amplification primers pA (5′-AGAGTTTGATCCTGGC-TCAG, positions 8–27, *Escherichia coli* numbering) and pH* (5′-AAGGAGGTGATCCAGCCGCA, positions 1541–1522, *E. coli* numbering) as described previously (Zamora *et al.*, 2012), and subjected to comparative analyses. The strains formed two separate phylogenetic groups represented by strains 435-08^T and 47B-3-09 (group A), and strains 412R-09^T and 60B-3-09 (group B). Strains displayed greater than 99.8–99.9 % 16S rRNA gene sequence similarity within their respective groups, suggesting a close genealogical identity. The identification of the phylogenetic neighbours and calculation of pair-wise 16S rRNA gene sequence similarities were achieved using the EzTaxon server (Kim *et al.*, 2012; http://eztaxon-e.ezbiocloud.net/). The 16S rRNA gene sequences of the type strains of all species with validly published names of the genus *Flavobacterium* were retrieved from GenBank and aligned with the newly determined sequences using the program SeqTools (Rasmussen, 2002). Strains 435-08^T and 412R-09^T exhibited 98.3 % 16S rRNA gene sequence similarity. Sequence searches showed that both groups of strains were most closely related to members of the genus *Flavobacterium*. The 16S rRNA gene sequence of strains 435-08^T and 412R-09^T exhibited the highest levels of similarity with the type strains of *Flavobacterium aquidurense* WB-1.1.56^T (98.6 % sequence similarity) and *Flavobacterium frigidimaris* KUC-1^T (98.9 % sequence similarity), respectively. In addition, strains 435-08^T and 412R-09^T exhibited 16S rRNA gene sequence similarities greater than 97.0 % with 12 and 19 other species of the genus *Flavobacterium*, respectively (Table 1).

Phylogenetic trees were reconstructed, according to three different algorithms: neighbour-joining (Saitou & Nei, 1987), using the programs SeqTools and TreeView (Page, 1996; Rasmussen, 2002); maximum-likelihood, using the PHYML software (Guindon & Gascuel, 2003); and maximum-parsimony, using the software package MEGA version 4 (Kumar *et al.*, 2004). Genetic distances for the neighbour-joining and maximum-likelihood algorithms were calculated by the Kimura two-parameter model (Kimura, 1980) and close-neighbour-interchange (search level=2, random additions=100) was applied in the maximum-parsimony analysis. The stability of the groupings was estimated by bootstrap analysis (1000 replications). The reconstructed phylogenetic tree, based on the neighbour-joining algorithm (Fig. 1), as well as two other methods (not shown), revealed a clear affiliation of both groups of trout strains to the genus *Flavobacterium*. It is evident from the phylogenetic tree (Fig. 1), that the strains formed two distinct sublineages, clustering with a subgroup of three species that included *F. aquidurense*, *F. araucananum* and *F. frigidimaris*. However, the position of the two strains within this subgroup was not supported by significant bootstrap value. The GenBank accession numbers for the 16S rRNA gene sequences of trout strains sequenced in this study are shown in Fig. 1.

Among species of the genus *Flavobacterium*, 16S rRNA gene sequence similarities lower than 98.0 % always correlate with levels of genomic DNA–DNA relatedness lower than 70 % (Lim *et al.*, 2011; Xu *et al.*, 2011; Yoon *et al.*, 2011; Chen *et al.*, 2013; Kämpfer *et al.*, 2012). For that reason, DNA–DNA hybridizations were carried out only between strains 435-08^T and 412R-09^T and the type strains of their respective most closely phylogenetically related species with 16S rRNA gene sequence similarities greater than 98.0 %. Genomic DNA–DNA hybridizations were carried out between strains 435-08^T and 47B-3-09 (group A), between strains 412R-09^T and 60B-3-09 (group B), and between strains 435-08^T or 412R-09^T and the type strains of their respective phylogenetically closely related species. The type strains *F. oncorhynchi* 631-08^T, *F. aquidurense* WB-1.1.56^T, *F. araucananum* LM-19-Fp^T, *F. psychrolimnae* LMG 22018^T, *F. pectinovorum* DSM 6368^T, *F. saccharophilum* DSM 1811^T, *F. frigidimaris* KUC-1^T and *F. chungangense* CJ7^T were included in this study. DNA was extracted and purified by the method of Marmur (1961). Hybridization studies were carried out using the membrane method of Johnson (1994) described in detail by Arahal *et al.* (2001). The hybridization experiments were carried out under optimal conditions, at a temperature of 44 °C, which is within the limits of validity for the

Table 1. DNA–DNA hybridization data and 16S rRNA gene sequence similarities of *F. tructae* sp. nov. and *F. piscis* sp. nov. with related species of the genus *Flavobacterium*

Strain	Strain 435-08^T		Strain 412R-09^T	
	16S rRNA gene similarity (%)	DNA–DNA hybridization (%)	16S rRNA gene similarity (%)	DNA–DNA hybridization (%)
F. aquidurense WB-1.1.56^T	98.6	14.6±3.6	98.5	28.2±1.8
F. araucananum LM-19-Fp^T	98.5	33.4±2.1	98.5	38.1±1.5
F. frigidimaris KUC-1^T	98.4	38.8±1.2	98.9	55.0±1.3
Flavobacterium oncorhynchi 631-08^T	98.2	23.7±1.9	97.8	ND
F. chungangense CJ7^T	98.1	30.4±1.7	97.8	ND
F. pectinovorum DSM 6368^T	97.9	ND	98.7	45.4±1.5
F. hibernum ATCC 51468^T	97.8	ND	97.5	ND
F. hercynium WB 4.2-33^T	97.5	ND	97.7	ND
F. psychrolimnae LMG 22018^T	97.5	ND	98.2	53.1±1.9
F. resistens BD-b365^T	97.5	ND	97.5	ND
F. saccharophilum DSM 1811^T	97.3	ND	98.1	37.7±3.8
F. hydatis DSM 2063^T	97.2	ND	97.8	ND
F. glaciei 0499^T	97.0	ND	97.0	ND
F. granuli Kw05^T	97.0	ND	97.4	ND
F. succinicans DSM 4002^T	<97.0	ND	97.6	ND
F. fryxellicola LMG 22022^T	<97.0	ND	97.3	ND
F. limicola ST-82^T	<97.0	ND	97.3	ND
F. omnivorum JCM 11313^T	<97.0	ND	97.2	ND
F. tiangeerense 0563^T	<97.0	ND	97.2	ND
F. micromati LMG 21919^T	<97.0	ND	97.1	ND
F. aquatile ATCC 11947^{T*}	<97.0	ND	<97.0	ND

ND, Not determined.
**F. aquatile* ATCC 11947^T, representing the type species of the genus *Flavobacterium*, has also been included.

membrane method (De Ley & Tijtgat, 1970). The percentages of hybridization were calculated as described by Johnson (1994). Three independent determinations were carried out for each experiment and the results reported as mean values. DNA–DNA hybridizations between strains 435-08^T and 47B-3-09 and between strains 412R-09^T and 60B-3-09 yielded binding values of 87.4 and 92.9 %, respectively. The DNA–DNA hybridization value between strains 435-08^T and 412R-09^T was 36.3 %. The levels of DNA–DNA relatedness for strain 435-08^T with respect to *F. oncorhynchi* 631-08^T, *F. aquidurense* WB-1.1.56^T, *F. araucananum* LM-19-Fp^T, *F. frigidimaris* KUC-1^T and *F. chungangense* CJ7^T ranged between 14.6 and 38.8 % (Table 1). The DNA–DNA hybridization values for strain 412R-09^T with respect to *F. frigidimaris* KUC-1^T, *F. pectinovorum* DSM 6368^T, *F. aquidurense* WB-1.1.56^T, *F. psychrolimnae* LMG 22018^T, *F. araucananum* LM-19-Fp^T and *F. saccharophilum* DSM 1811^T ranged between 28.2 and 55.0 % (Table 1). These data clearly confirmed that strains of groups A and B belong to two distinct genomic species (Wayne *et al.*, 1987; Rosselló-Mora, 2006).

The G+C contents of the genomic DNA of two representative strains (435-08^T and 412R-09^T) were determined from the mid-point value (T_m) of the thermal denaturation profile (Marmur & Doty, 1962), obtained with a Perkin-Elmer UV-Vis Lambda 20 spectrophotometer at 260 nm. The DNA G+C contents of strains 435-08^T and 412R-09^T were 36.2 and 34.3 mol%, respectively, values consistent with those of members of the genus *Flavobacterium* (Bernardet *et al.*, 1996; Bernardet & Bowman, 2006).

Fatty acid composition, respiratory quinones and polar lipids were determined in strains 435-08^T and 421R-09^T. Analysis of polar lipids and respiratory quinones was carried out by the Identification Service, DSMZ, Braunschweig, Germany. The major quinone in both strains was MK-6 (96 % in strain 435-08^T and 94 % in strain 412R-09^T). Both strains also contained minor amounts of MK-5 (4 % in strain 435-08^T and 6 % in strain 412R-09^T). These data are in accordance with those reported for members of the family *Flavobacteriaceae* (Bernardet & Nakagawa, 2006). The polar lipid profile of strain 435-08^T consisted of phosphatidylethanolamine, an unknown aminolipid, lipids L1, L2 and L3 and an unknown glycolipid (Fig. S1a, available in IJSEM Online) while the polar lipid profile of strain 412R-09^T consisted of a similar pattern except for the absence of glycolipid (Fig. S1b). The cell fatty acid-fatty acid methyl ester (CFA-FAME) analyses was done with cultures on Columbia II agar base (4397596; BBL) with 5 % horse blood, at 30 °C for 30–48 h, under aerobic conditions. The CFA-FAME profile

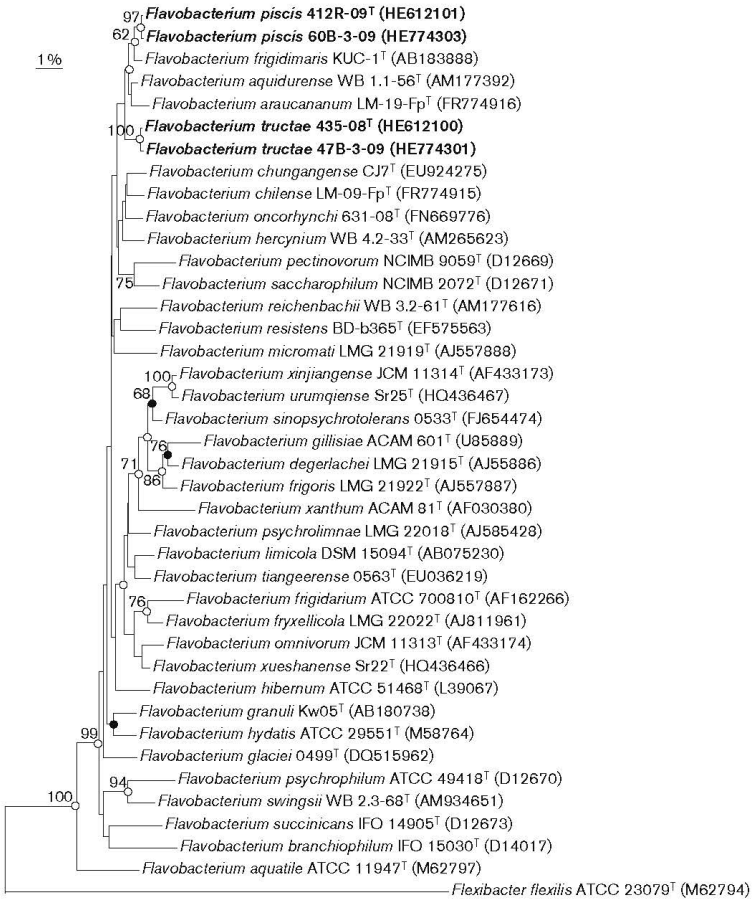

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequence comparisons, obtained with the neighbour-joining algorithm, showing the relationships of *Flavobacterium tructae* sp. nov. and Flavobacterium *piscis* sp. nov. with related species. Bootstrap values (expressed as a percentage of 1000 replications) >70 % are included, at the branching points. Filled circles indicate that the corresponding nodes (groupings) are also obtained in the maximum-likelihood tree. Open circles indicate that the corresponding nodes (groupings) are also obtained in the maximum-likelihood and parsimony trees. *Flexibacter flexilis* ATCC 23079^T (M62794) was used as an outgroup. Bar, 1 % sequence divergence.

was determined using GC (HP 5890; Hewlett Packard) and a standardized protocol similar to that of the MIDI Sherlock MIS system (Sasser, 2001), described previously (Zamora *et al.*, 2012). CFAs were identified and the relative amounts were expressed as percentages of the total fatty acids of the strains. The predominant CFAs of strain 435-08^T were iso-$C_{15:0}$ (31 %), $C_{16:1}\omega7c$ (17 %) and $C_{15:0}$ (12 %) while those of strain 412R-09^T were iso-$C_{15:0}$ (20 %) and $C_{16:1}\omega7c$ (17 %). Other CFAs were observed in percentages between 7 and 1 % (Table 2). These results corroborate the adscription of the trout strains to the genus *Flavobacterium*.

The minimal standards for the description of new taxa in the family *Flavobacteriaceae* were followed for the phenotypic characterization of the strains (Bernardet *et al.*, 2002). Gram-staining was performed as described by Smibert & Krieg (1994). Oxidase activity was determined by monitoring the oxidation of tetramethyl-*p*-phenylenediamine on

Table 2. Cell fatty acid (CFA) profiles of *Flavobacterium tructae* sp. nov. and *Flavobacterium piscis* sp. nov. isolated from trout

Strains: 1, *F. tructae* sp. nov. 435-08^T; 2, *F. tructae* sp. nov. 47B-3-09; 3, *F. piscis* sp. nov. 412R-09^T; 4, *F. piscis* sp. nov. 60B-3-09; 5, *F. araucananum* LM-19-Fp^T; 6, *F. aquidurense* WB-1.1.56^T; 7, *F. frigidimaris* KUC-1^T; 8, *F. aquatile* ATCC 11947^T (representing the type species of the genus *Flavobacterium*). CFAs with contents greater than 1.0 % of total CFAs are indicated. Values are percentages of total CFAs. TR, Trace amount, i.e. <1.0 %; ECL, equivalent chain length. Data are taken from this study and from the CCUG database (http://www.ccug.se). All strains were cultivated under the same conditions.

Fatty acid	ECL	1	2	3	4	5	6	7	8
Unknown fatty acid	11.541	TR	TR	2	2	TR	TR	TR	2
Unknown fatty acid	12.555	TR	TR	2	1	–	TR	–	1
$C_{14:0}$ aldehyde	12.931	TR	1	1	2	–	–	–	1
iso-$C_{15:0}$ aldehyde	13.566	1	2	2	1	1	1	1	2
iso-$C_{14:0}$	13.618	–	–	1	–	–	–	–	–
anteiso-$C_{15:0}$ aldehyde	13.647	–	–	–	–	–	–	–	2
$C_{14:0}$	14.000	2	2	1	2	TR	–	1	TR
iso-$C_{15:1}$ G	14.441	4	2	7	2	7	2	4	4
iso-$C_{15:0}$	14.621	31	33	20	25	28	25	28	9
anteiso-$C_{15:0}$	14.711	TR	TR	2	2	4	1	3	12
Unknown fatty acid	14.809	–	–	–	–	–	–	–	1
$C_{15:1}\omega6c$	14.856	5	6	5	7	5	11	4	5
$C_{15:0}$	15.000	12	12	6	9	7	13	5	9
Summed feature 1*	15.485	1	2	–	–	–	–	–	–
iso-$C_{16:0}$	15.626	1	TR	1	TR	1	TR	1	TR
$C_{16:1}\omega7c$	15.819	17	13	17	18	18	7	19	22
$C_{16:1}\omega5c$	15.908	TR	TR	1	1	–	–	–	–
$C_{16:0}$	16.000	5	3	2	3	2	1	2	2
iso-$C_{15:0}$ 3-OH	16.135	6	7	7	7	6	8	8	4
$C_{15:0}$ 2-OH	16.217	–	–	–	–	–	–	–	2
iso-$C_{17:1}\omega9c$	16.416	2	2	3	2	6	5	4	1
Summed feature 2*	16.481	–	–	–	–	1	–	2	–
$C_{15:0}$ 3-OH	16.504	1	1	1	2	–	4	–	–
anteiso-$C_{17:1}\omega9c$	16.525	–	–	–	–	–	–	–	2
$C_{17:1}\omega8c$	16.792	–	–	–	–	TR	TR	–	1
$C_{17:1}\omega6c$	16.862	TR	TR	4	2	3	5	3	4
iso-$C_{16:0}$ 3-OH	17.145	TR	TR	2	1	TR	1	2	1
$C_{16:0}$ 3-OH	17.520	5	4	4	6	1	TR	3	3
iso-$C_{17:0}$ 3-OH	18.161	4	4	6	5	5	7	6	5

*Summed features represent a set of more than one CFA that could not be distinguished. Summed feature 1 comprised $C_{14:0}$ 3-OH/iso-$C_{16:1}$ I and summed feature 2 comprised iso-$C_{17:1}$ I/DMA $C_{16:0}$.

filter paper and catalase activity was determined using 3 % H_2O_2 solution (Smibert & Krieg, 1994). Hydrolysis of L-tyrosine (0.5 %, w/v), lecithin (5 %, w/v) (Smibert & Krieg, 1994), aesculin (0.01 % aesculin and 0.05 % ferric citrate, w/v), gelatin (4 %, w/v), starch (0.2 %, w/v) and casein (50 % skimmed milk, v/v; Difco) were tested using nutrient agar (Smibert & Krieg, 1994; Tindall *et al.*, 2008). DNase test agar (Difco) was used for the DNase assay. Hydrolysis of urea (1 %, w/v) was tested as described by Bowman *et al.* (1996). Growth in brain heart infusion broth was assessed at 15, 25, 30, 37 and 42 °C, with 3.0, 4.5 and 6.5 % added NaCl, and under anaerobic (with 4–10 % CO_2) and microaerobic (with 5–15 % O_2 and 5–12 % CO_2) conditions, using GasPak Plus and CampyPak Plus sytems (BBL), respectively. Growth was tested on MacConkey (bioMérieux), nutrient (Difco) and trypticase-soy (bioMérieux) agar plates. The presence of gliding motility was determined using the hanging drop technique, and the production of flexirubin-type pigments and extracellular glycans was assessed using the KOH and Congo red tests, respectively (Bernardet *et al.*, 2002). The strains were further biochemically characterized using the API 20NE and API ZYM systems (bioMérieux) according to the manufacturer's instructions, except that the incubation temperature was 25 °C. *F. aquidurense* WB-1.1.56^T, *F. araucananum* LM-19-Fp^T and *F. frigidimaris* KUC-1^T were included in this study as references for the investigation of the phenotypic properties of the trout strains, using the same laboratory conditions. Strains of the phylogenetic group A (435-08^T and 47B-3-09) showed identical physiological and biochemical characteristics. Also,

strains of the phylogenetic group B (412R-09^T and 60B-3-09) exhibited the same physiological and biochemical features. Strains of groups A and B exhibited different physiological and biochemical characteristics. A detailed description of the morphological, physiological and biochemical characteristics of each group is given the species description. The phenotypic characteristics that differentiated strains of groups A and B from phylogenetically related phylogenetic species are shown in Table 3. Strains of group A can be readily differentiated from *F. aquidurense* by their ability to reduce nitrate to nitrite. Additionally, *F. aquidurense* hydrolyses urea but not gelatin and uses arabinose as sole carbon source but not citrate, while the new strains give opposite results for these tests. Strains of group B can be differentiated from their close phylogenetic neighbour, *F. frigidimaris*, because this species grows in marine agar but not at 30 °C. Moreover, the novel species reduced nitrate to nitrite, did not produce cystine arylamidase or β-glucosidase, and was able to hydrolyse gelatin. Moreover, the two novel species can be differentiated from the clinically relevant fish pathogens *F. columnare*, *F. psychrophilum* and *F. branchiophilum* by the inability of these three species to grow in trypticase-soy and to hydrolyse aesculin (Bernardet & Bowman, 2006). Other species isolated from diseased fish such as *F. hydatis*, F. johnsoniae and *F. succinicans* are motile (gliding), degrade DNA and produce acid from carbohydrates (Bernardet & Bowman, 2006), while both novel species give opposite results for these tests. Moreover, the two novel species can readily be differentiated from *F. chilense* and *F. araucananum*, because the latter species are motile (gliding), grow with 3 % NaCl and assimilate mannitol (Kämpfer *et al.*, 2012), and from *F. oncorhynchi*, which does not hydrolyse gelatin and produces β-galactosidase while both novel species give opposite results for these tests (Zamora *et al.*, 2012).

Table 3. Characteristics that differentiate *Flavobacterium tructae* sp. nov. and *Flavobacterium piscis* sp. nov. from closely related species of the genus *Flavobacterium* based on the 16S rRNA gene sequence similarity

Strains: 1, *F. tructae* sp. nov. 435-08^T; 2, *F. piscis* sp. nov. 412R-09^T; 3, *F. araucananum* LM-19-Fp^T; 4, *F. aquidurense* WB-1.1.56^T; 5, *F. frigidimaris* KUC-1^T; 6, *F. aquatile* ATCC 11947^T (representing the type species of the genus *Flavobacterium*). Data are from this study. +, Positive reaction; −, negative reaction.

Characteristic	1	2	3	4	5	6
Growth on Marine agar	−	−	−	−	+	−
Growth at 30 °C	+	+	+	+	−	+
Degradation of L-tyrosine	+	−	−	+	−	+
Nitrate reduction	+	+	+	−	−	+
Hydrolysis of:						
Urea	−	−	−	+	−	+
Gelatin	+	+	−	−	−	−
Assimilation of:						
Arabinose	−	+	+	+	+	−
Mannitol	−	−	−	−	+	−
Citrate	+	−	−	−	−	−
Production of:						
Valine arylamidase	+	−	+	+	+	−
α-Galactosidase	−	+	−	−	−	−
α-Glucosidase	−	+	+	−	+	+
β-Glucosidase	−	−	+	−	+	−
N-Acetyl-β-glucosaminidase	−	+	−	−	+	−

Overall, the results of the present polyphasic study demonstrate that the characteristics of the new strains from diseased rainbow trout match those described for the genus *Flavobacterium* and these strains should be assigned to this genus. The phylogenetic, genotypic and phenotypic differences clearly demonstrate that strains of groups A and B merit classification as two novel species, for which the names *Flavobacterium tructae* sp. nov. and *Flavobacterium piscis* sp. nov. are proposed.

The four strains from diseased trout were characterized by pulsed-field gel electrophoresis (PFGE) profiling of their genomic DNA, after digestion with the restriction enzyme *Xho*I, according to previous specifications (Chen *et al.*, 2008). Similarities between restriction endonuclease digestion profiles were based on visual comparisons of the band patterns of strains run in the same gel. Strains differing in at least one band were considered different. Strains of group A (435-08^T and 47B-3-09) displayed different PFGE restriction profiles (Fig. S2). Strains of group B (412R-09^T and 60B-3-09) also exhibited two different PFGE profiles. The virulence of the new strains has not been determined yet by challenge experiments.

Description of *Flavobacterium tructae* sp. nov.

Flavobacterium tructae (truc'tae L. gen. n. *tructae* of a trout, from which the type strain was isolated).

Cells are Gram-staining-negative rods, 0.3 μm wide by 3 μm long, non-endospore-forming and non-gliding. Strains grow well under aerobic conditions and grow weakly under micro-aerobic conditions. Strains grow at 15–30 °C with optimal growth at approximately 25 °C, while no growth occurs at 37 °C or at 42 °C. Growth occurs on trypticase-soy and nutrient agars but not on marine agar after incubation at 25 °C for 72 h. Growth does not occur in brain heart infusion broth containing 3, 4.5 or 6.5 % NaCl. Colonies are circular, yellow-pigmented, smooth and entire on TGE agar after 72 h of incubation at 25 °C. Colonies are non-haemolytic on Columbia agar after 72 h of incubation at 25 °C. Diffusible flexirubin-type pigments are produced. Congo red is not absorbed by colonies. Catalase and oxidase are produced. Nitrate and nitrite are reduced. Starch, tyrosine, gelatin and casein are degraded but DNA and agarose are not. A brown pigment is not produced on tyrosine agar. Aesculin is hydrolysed

but not urea, lecithin or arginine. Indole and H_2S are not produced. Acid is not produced from D-glucose. Glucose, mannose, *N*-acetylglucosamine, maltose and citrate are used as sole carbon and energy sources but not arabinose, mannitol, gluconate, caprate, adipate or malate. Activities of alkaline phosphatase, leucine arylamidase, valine arylamidase, acid phosphatase and naphthol-AS-BI-phophohydrolase are detected. Esterase C4, β-galactosidase, esterase lipase C8, lipase C14, α-glucosidase, cystine arylamidase, α-chymotrypsin, trypsin, α-galactosidase, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are not detected. Cells contain menaquinone-6 (MK-6) as the major respiratory quinone. The major cellular fatty acids are iso-$C_{15:0}$, $C_{16:1}\omega7c$ and $C_{15:0}$.

The type strain, 435-08^T (=CECT 7791^T=CCUG 60100^T), was isolated from the liver of a rainbow trout. The DNA G+C content of this strain is 36.2 mol%.

Description of *Flavobacterium piscis* sp. nov.

Flavobacterium piscis (pis'cis. L. gen. masc. n. *piscis* of a fish).

Cells are Gram-staining-negative rods, 0.1 μm wide by 1.7 μm long, non-endospore-forming and non-gliding. Strains grow well under aerobic conditions and grow weakly under micro-aerobic conditions. Strains grow at 15–30 °C with optimal growth at approximately 25 °C, while no growth occurs at 37 °C or at 42 °C. Growth occurs on trypticase-soy and nutrient agars but not on marine agar after incubation at 25 °C for 72 h. Growth does not occur in brain heart infusion broth containing 3, 4.5 and 6.5 % NaCl. Colonies are circular, yellow-pigmented, smooth and entire on TGE agar after 72 h of incubation at 25 °C. Colonies are non-haemolytic on Columbia agar after 72 h of incubation at 25 °C. Diffusible flexirubin-type pigments are produced. Congo red is not absorbed by colonies. Catalase and oxidase are produced. Nitrate and nitrite are reduced. Starch, gelatin and casein are degraded but tyrosine, DNA and agarose are not. Aesculin is hydrolysed but not lecithin, urea, arginine or DNA. Indole and H_2S are not produced. Acid is not produced from D-glucose. Glucose, arabinose, mannose, *N*-acetylglucosamine and maltose are used as sole carbon and energy sources but not mannitol, gluconate, caprate, adipate, citrate or malate. Activities for alkaline phosphatase, leucine arylamidase, naphthol-AS-BI-phophohydrolase, acid phosphatase, α-galactosidase, α-glucosidase and *N*-acetyl-β-glucosaminidase are detected. Esterase C4, esterase lipase C8, β-galactosidase valine arylamidase, lipase C14, cystine arylamidase, α-chymotrypsin, trypsin, β-glucuronidase, β-glucosidase, α-mannosidase and α-fucosidase are not detected. Cells contain menaquinone-6 (MK-6) as the major respiratory quinone. The major cellular fatty acids are iso-$C_{15:0}$ and $C_{16:1}\omega7c$.

The type strain, 412R-09^T (=CECT 7911^T=CCUG 60099^T), was isolated from the kidney of a rainbow trout. The DNA G+C content of this strain is 34.3 mol%.

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Characterization of flavobacteria possibly associated with fish and fish farm environment. Description of three novel *Flavobacterium* species: *Flavobacterium collinsii* sp. nov., *Flavobacterium branchiarum* sp. nov., and *Flavobacterium branchiicola* sp. nov.

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ABSTRACT

Members of the genus *Flavobacterium* are widely distributed microorganisms that can be recovered from a wide variety of environments. There has been an increase in the number of species isolated from fish or fish farm environments, some of them possibly associated with disease. Fourteen *Flavobacterium*-like isolates recovered from gills, surface of eggs and liver of rainbow trout were characterized by a polyphasic study that included a deep phenotypic, chemotaxonomic, genotypic and phylogenetic analysis. Comparative 16S rRNA gene analysis revealed that isolates represented three separate phylogenetic groups (A–C) within the genus *Flavobacterium*. Further genotypic and phenotypic data demonstrated that they represent three novel species of the genus *Flavobacterium* for which the names *Flavobacterium collinsii*, *Flavobacterium branchiarum* and *Flavobacterium branchiicola* are proposed. Most isolates of the three species were collected from gills indicating that they are probably commensal organisms that are able to colonize this tissue. However, some strains were recovered from the liver of two different trout, suggesting their ability to infect trout.

1. Introduction

Members of the genus *Flavobacterium* comprise a group of physiologically diverse and widely distributed environmental microorganisms that have been isolated from many different habitats, including soil, water, sludge, plants, and food products such as fish, meat, poultry, milk and lactic acid beverages (Bernardet and Bowman, 2006; Bernardet and Nakagawa, 2006). In particular, flavobacteria are commonly isolated from fresh water environments related with aquaculture activities (Bernardet and Bowman, 2006) as well as from the skin, gills and eggs of fish (Bernardet and Bowman, 2011). Although several species are pathogenic for humans and different warm-blooded animals, the main concern of flavobacteria as pathogenic bacteria is associated with the ability of some species to cause disease in aquaculture facilities (Bernardet and Bowman, 2006). *Flavobacterium columnare*, *Flavobacterium branchiophilum* and *Flavobacterium psychrophilum* are well-recognized fish pathogens responsible for serious economic losses in the fish farming industry (Roberts, 2012; Starliper and Schill, 2012). Several other species such as *Flavobacterium hydatis*, *Flavobacterium johnsoniae*, *Flavobacterium succinicans*, *Flavobacterium chilense*, *Flavobacterium araucananum* and *Flavobacterium oncorhynchi* have also been occasionally associated with infections in fish (Bernardet and Bowman, 2006, 2011; Bernardet et al., 1996; Flemming et al., 2007; Kämpfer et al., 2012; Zamora et al., 2012). This plethora of flavobacteria associated with fish probably reproduces the diversity of the *Flavobacterium* species present in fish farm environments. Some of these species have been mainly isolated from external lesions of fish, from the surface of fish eggs or from the skin, gills and guts of fish, suggesting that they could be saprophytic or commensal organisms that may act as opportunistic fish pathogens (Bernardet and Bowman, 2006). Therefore, it is important to identify those *Flavobacterium* species isolated from fish or the environment where they are grown. In the present work, we present the results of a deep phenotypic and genetic characterization of 14 *Flavobacterium*-like isolates mainly recovered from the gills and eggs of farmed trout. The outcome of this study is the description of three new *Flavobacterium* species.

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2. Material and methods

2.1. Isolation of bacteria

Fourteen Gram-negative, rod-shaped bacteria were recovered from liver (isolates 983-08^T and 991-08) and gills (isolates 978B-08, 53B-3-09, 55B-09, 56B-1-09, 57B-2-09^T, 58B-1-09, 59B-3-09^T, 60B-4-09, 61B-09, 62B-2-09) of twelve different trout (*Oncorhynchus mykiss*) in the same fish farm. Trout isolates were recovered from four different ponds (978B-08, 983-08^T and 991-08 from pond 1; 53B-3-09, 55B-09, 56B-1-09 and 59B-3-09^T from pond 2; 57B-2-09^T, 58B-1-09 and 60B-4-09 from pond 3; 61B-09, 62B-2-09 from pond 4) in the same fish farm, in two different years (2008 and 2009). Two additional isolates (977H-09 and 976H-09) were recovered from the surface of fish eggs. The isolates were obtained on tryptone glucose extract (TGE) agar (Difco) after incubation at 25 °C for 72 h under aerobic conditions. Isolates were stored at −80 °C in a cryopreserving medium until further use.

2.2. Phylogenetic analysis

To establish the phylogenetic allocation of the bacteria, a large continuous fragment (approximately 1400 bases) of the 16S rRNA gene of the fourteen isolates was obtained in both directions, using the universal amplification primer: pA (5′-AGAGTTTGATCCTGGCTCAG, positions 8–27, *Escherichia coli* numbering) and pH* (5′-AAGGAGGTGATCCAGCCGCA, positions 1541–1522, *E. coli* numbering) as described previously (Zamora et al., 2012), and subjected to a comparative analysis. The identification of the phylogenetic neighbors and calculations of pair-wise 16S rRNA gene sequence similarities were achieved using the EzTaxon, server [http://eztaxon-e.ezbiocloud.net/Kim et al., 2012]. The 16S rRNA gene sequences of the type strains of all species with valid names of the genus *Flavobacterium* were retrieved from GenBank and aligned with the newly determined sequences using the program SeqTools (Rasmussen, 2002). Phylogenetic trees were constructed according to three different algorithms: neighbor-joining (Saitou and Nei, 1987), using the programs SeqTools and TreeView (Page, 1996); maximum-likelihood, using the PhyML software (Guindon and Gascuel, 2003); and maximum-parsimony, using the software package MEGA (Molecular Evolutionary Genetics Analysis) version 4 (Tamura et al., 2011). Genetic distances for the neighbor-joining and the maximum-likelihood algorithms were calculated by the Kimura two-parameter (Kimura, 1980) and close-neighbor-interchange (search level = 2, random additions = 100) was applied in the maximum-parsimony analysis. The stability of the groupings was estimated by bootstrap analysis (1000 replications).

2.3. Genotypic analysis

Genomic DNA–DNA hybridizations were carried out between isolates 983-08^T, 978B-08, 977H-09, 991-08, 976H-09 and 53B-3-09 (group A), between isolates 57B-2-09^T, 55B-09, 61B-09, 62B-2-09 and 56B-1-09 (group B) and between isolates 59B-3-09^T, 60B-4-09 and 58B-1-09 (group C). Genomic DNA–DNA hybridizations were also carried between isolates 983-08^T, 57B-2-09^T, 59B-3-09^T and the type strains of their respective closet phylogenetically related species. DNA was extracted and purified by the method of Marmur (1961). Hybridization studies were carried out, using the membrane method of Johnson (1994), described in detail by Arahal et al. (2001). The hybridization experiments were carried out under optimal conditions, at a temperature of 44 °C, which is within the limits of validity for the membrane method (de Ley and Tijtgat, 1970). The percentages of hybridization were calculated as described by Johnson (1994). Three independent determinations were carried out for each experiment and the results reported as mean values. The type strains of species *Flavobacterium aquidurense* WB-1.1.56^T, *F. araucananum* LM-19-Fp^T, *Flavobacterium pectinovorum* DSM 6368^T, *F. hydatis* DSM 2063^T, *Flavobacterium granuli* Kw05^T, *Flavobacterium saccharophilum* DSM 1811^T, *Flavobacterium frigidimaris* KUC-1^T and *Flavobacterium chungangense* CJ7^T were included in this study.

The G + C contents of the genomic DNA of three representative isolates (983-08^T, 59B-3-09^T and 57B-2-09^T) were determined from the mid-point value (Tm) of the thermal denaturation profile (Marmur, 1961), obtained with a Perkin-Elmer UV–Vis Lambda 20 spectrophotometer at 260 nm.

2.4. Chemotaxonomic analysis

Fatty acid composition and respiratory quinones were determined in the isolates 57B-2-09^T, 59B-3-09^T and 983-08^T. For cell fatty acid–fatty acid methyl ester (CFA–FAME) analyses, isolates were grown on Columbia II agar base (BBL 4397596) with 5% horse blood, at 30 °C for 30–48 h, under aerobic conditions. The CFA-FAME profile was determined using gas-chromatography (Hewlett Packard HP 5890) and a standardized protocol similar to that of the MIDI Sherlock MIS system (Sasser, 2001), described previously (Zamora et al., 2012). CFAs were identified and the relative amounts were expressed as percentages of the total fatty acids of the isolates. The analysis of polar lipids and respiratory quinones was carried out by Identification Service and Dr. Brian Tindall, DSMZ, Braunschweig, Germany.

2.5. Physiological and biochemical characterization

The minimal standards for the description of new taxa in the family Flavobacteriaceae were followed for the phenotypic characterization of the isolates (Bernardet et al., 2002). Gram-staining was performed as described by Smibert and Krieg (1994). Oxidase activity was determined by monitoring the oxidation of tetramethyl-p-phenylenediamine on filter paper and catalase activity was determined, using 3% H_2O_2 solution (Smibert and Krieg, 1994). Hydrolysis of L-tyrosine (0.5%, w/v), lecithin (5%, w/v) (Smibert and Krieg, 1994), esculin (0.01% esculin and 0.05% ferric citrate, w/v), gelatine (4%; w/v), starch (0.2%, w/v), and casein [50% skimmed milk (Difco), v/v] were tested using nutrient agar (Bowman et al., 1996). DNase test agar (Difco) was used for the DNase assay. Hydrolysis of urea (1%, w/v) was tested as described by Bowman et al. (1996). Growth in the brain heart infusion broth was assessed at 15, 25, 30, 37 and 42 °C, with 3.0, 4.5 and 6.5% added NaCl, and under anaerobic (with 4–10% CO2) and micro-aerobic (with 5–15% O2 and 5–12% CO2) conditions, using GasPak Plus and CampyPak Plus sytems (BBL), respectively. Growth was tested on MacConkey (bioMérieux), nutrient (Difco) and trypticase-soy (bioMérieux) agar plates. The presence of gliding motility, using the hanging drop technique, the production of flexirubin-type pigments and extracellular glycans were assessed, using the KOH and Congo red tests, respectively (Bernardet et al., 1996). The isolates were further biochemically characterized using the API 20NE and API Zym systems (bioMérieux) according to the manufacturer's instructions, except that incubation temperature was 25 °C. The type strains of species *F. aquidurense* WB-1.1.56^T, *F. araucananum* LM-19-Fp^T, *F. pectinovorum* DSM 6368^T, *F. hydatis* DSM 2063^T, *F. granuli* Kw05^T, *F. saccharophilum* DSM 1811^T, *F. frigidimaris* KUC-1^T *and F. chungangense* CJ7^T were included in this study as references for the investigation of the phenotypic properties of the novel isolates, using the same laboratory conditions.

2.6. PFGE typing

The fourteen isolates were characterized by pulsed-field gel electrophoresis (PFGE) profiling of their genomic DNAs, after digestion with the restriction enzyme *XhoI*, according to previous specifications (Chen et al., 2008). Similarities between restriction endonuclease digestion profiles were based on visual comparisons of the band patterns of

isolates which run in the same gel. Isolates differing in at least one band were considered different.

3. Results and discussion

Comparative 16S rRNA gene analysis of the sequences of the 14 isolates showed that all isolates were most closely related to the members of the genus *Flavobacterium* and formed three separate phylogenetic groups (A–C), represented by isolates 976H-09, 977H-09, 978B-08, 983-08^T, 991-08 and 53B-3-09 (group A), isolates 55B-09, 61B-09, 62B-2-09, 56B-1-09, 57B-2-09^T (group B) and 58B-1-09, 59B-3-09^T, 60B-4-09 (group C). Within their respective groups, isolates of group A and C displayed 16S rRNA sequence similarities of between 99.9–100% and 99.8–99.9%, respectively. The 5 isolates of group B exhibited 100% 16S rRNA sequence similarity. Strain 983-08^T exhibited 98.2% and 98.0% 16S rRNA gene sequence similarity with isolates 57B-2-09^{T}and 59B-3-09^T, respectively. Isolates 59B-3-09^T and 57B-2-09^T exhibited 97.4% 16S rRNA gene sequence similarity. These isolates exhibited the highest levels of 16S rRNA gene sequence similarity with the type strains of *F. pectinovorum* DSM 6368^T (98.5% sequence similarity), *F. hydatis* DSM 2063^T (98.9%) and *F. aquidurense* WB-1.1.56^T (98.7% sequence similarity), respectively. They also exhibited 16S rRNA gene sequence similarities greater than 97.0% with another sixteen, eleven and fifteen *Flavobacterium* species, respectively. The phylogenetic tree based on the neighbor-joining algorithm (Fig. 1), as well as two other methods (not shown), confirmed the affiliation of isolates of groups A–C to the genus *Flavobacterium*. It is evident from the phylogenetic tree (Fig. 1), that the isolates form three distinct sub-lineages. Isolates 57B-2-09^T and 983-08^T clustered with a sub-group of three species that included *F. hydatis*, *F. granuli* and *Flavobacterium hibernum* while the strain 59B-3-09^T clustered in a different sub-group of four species that included *F. frigidimaris*, *F. araucananum*, *F. aquidurense* and *Flavobacterium resistens* (Fig. 1). However, the position of these isolates within their sub-groups was not supported by significant bootstrap values. The GenBank accession numbers for the 16S rRNA gene sequences of novel isolates sequenced in this study are shown in Fig. 1.

DNA–DNA hybridizations between isolates of each group yielded reassociation values of 78.2–95.5%, 93.0–99.0% and 94.4–98.7%, for isolates of group A, B and C respectively. These values confirmed that isolates within their respective groups are members of the same species. *Flavobacterium* species with 16S rRNA gene sequence similarities below 98.0% correlate with levels of genomic DNA–DNA relatedness always lower than 70% (Lim et al., 2011; Xu et al., 2011; Yoon et al., 2011; Kämpfer et al., 2012; Zamora et al., 2012; Chen et al., 2013). For that reason, DNA–DNA hybridizations were carried out only between strain 983-08^T and isolates 57B-2-09^T and 59B-3-09^T and between isolates 983-08^T, 57B-2-09^T, 59B-3-09^T and the type strains of their respective closest phylogenetically related species with 16S rRNA gene sequence similarities greater than 98.0%. DNA–DNA hybridization between strain 983-08^T and isolates 57B-2-09^T and 59B-3-09^T showed reassociation values of 37.3% and 15.2%, respectively. DNA–DNA hybridization between strain 983-08^T and the type strains of *F. pectinovorum* DSM 6368^T, *F. saccharophilum* DSM 1811^T, *F. aquidurense* WB-1.1.56^T, *F. hydatis* DSM 2063^T and *F. frigidimaris* KUC-1^T showed relatedness ranging between 19.6% and 47.9%. The DNA–DNA hybridization values between strain 57B-2-09^T and type strains of *F. hydatis* DSM 2063^T and *F. granuli* Kw05^T were 30.9% and 46.8%, respectively. Also, the DNA–DNA hybridization reassociation values between strain 59B-3-09^T and the type strains of *F. aquidurense* WB-1.1.56^T, *F. frigidimaris* KUC-1^T, *F. chungangense* CJ7^T, *F. araucananum* LM-19-Fp^T, *F. pectinovorum* DSM 6368^T ranged between 27.5% and 54.5%. These values are below the 70% cut-off point for species delineation (Wayne et al., 1987; Stackkebrandt and Goebel, 1994) and confirmed that the group of trout isolates A–C belong to distinct genomic species of the genus *Flavobacterium*. The DNA G + C contents of isolates 983-08^T, 57B-2-

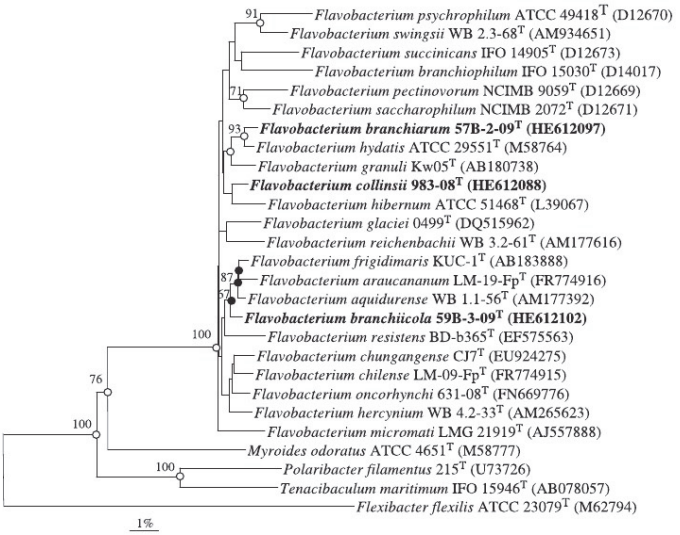

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequence comparisons, obtained with the neighbor-joining algorithm showing the relationships of *Flavobacterium branchiarum* sp. nov., *Flavobacteruium branchiicola* sp. nov. and *Flavobacteruium collinsii* sp. nov. with related species. Bootstrap values expressed as a percentage of tree. Open circles indicate that the corresponding nodes (groupings) and are also obtained on the maximum-likelihood and parsimony trees. *Flexibacter flexilis* ATCC 23079^T (M62794) was used as an outgroup. Bar, 1% sequence divergence.

09^T and 59B-3-09^T were 35.5, 35.4 and 33.6 mol%, respectively. These values are consistent with those of the genus *Flavobacterium* (Bernardet et al., 1996, 2002).

Analysis of whole-cell fatty acid of isolates 983-08^T, 57B-2-09^T and 59B-3-09^T revealed that iso-$C_{15:0}$ and $C_{16:1}$ *ω7c* were the predominant cell fatty acids in the three isolates (Table 1). Other CFAs were observed in percentages between 10% and 1% (Table 1). The major quinone in the three isolates was MK-6 (95% in isolates 983-08^T and 57B-2-09^T and 89% in strain 59B-3-09^T), but they also contained minor amounts of MK-5 (2% in strain 983-08^T, 5% in strain 57B-2-09^T and 11% in strain 59B-3-09^T). These chemotaxonomic data corroborated the adscription of the novel isolates to the genus *Flavobacterium* (Bernardet et al., 1996, 2002). The polar lipid profile of strain 983-08^T consisted of phosphatidylethanolamine, two unidentified aminolipids (AL1 and AL2), three unidentified lipids (L1, L2 and L3) and an unidentified glycolipid (Fig. 2A). The polar lipid profile of strain 57B-2-09^T was similar and consisted of phosphatidylethanolamine, three unidentified aminolipids (AL1, AL2 and AL3), three unidentified lipids (L1, L2 and L3) and an unidentified glycolipid (Fig. 2B); finally strain 59B-3-09^T also has phosphatidylethanolamine, the three unidentified lipids (L1, L2 and L3), and one unidentified glycolipid (GL) and one unidentified aminolipid (Fig. 2C).

Phenotypically all isolates of groups A–C exhibited the following characteristics: isolates gave circular, yellow-pigmented, smooth and entire colonies on TGE agar after 72 h of incubation at 25 °C. Cells were Gram-negative rods (0.1 to 0.3 μm wide by 2.3 to 3 μm long), non-endospore-forming, and non-gliding. Isolates grew well under aerobic conditions and grew weakly under micro-aerobic conditions. Isolates grew at 15–25 °C with optimal growth at approximately 22 °C, while no growth occurred either at 30 °C, 37 °C or at 42 °C. Growth occurred on trypticase-soy and nutrient agars but not on marine agar after incubation at 25 °C for 72 h. They did not grow in the brain heart infusion broth containing 3%, 4.5% or 6.5% NaCl. Colonies were non-haemolytic on Columbia agar after 72 h of incubation at 25 °C, did not produce diffusible flexirubin-type pigments and Congo red was not absorbed. They were catalase and oxidase positive. They degraded starch and casein but not gelatine, DNA or agarose. A brown pigment was not produced on tyrosine agar. Isolates hydrolyzed esculin but not lecithin or arginine. Indole and H_2S were not produced. Isolates did not produce acid from D-glucose. Glucose and mannose were used as sole carbon and energy sources but not gluconate, caprate, adipate, and malate. Isolates produced alkaline phosphatase, leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase, but not esterase C4, ester lipase C8, lipase C14, cystine arylamidase, α-chymotrypsin, α-galactosidase, β-glucuronidase, α-mannosidase and α-fucosidase.

Several morphological, physiological and biochemical characteristics can differentiate isolates of groups A–C. Thus, with the API 20NE system, isolates of the phylogenetic group A gave the numerical profile 0462305, isolates of the phylogenetic group B gave the numerical profile 0442305 and isolates of the phylogenetic group C gave the numerical

Table 1
Cellular fatty acid (CFA) compositions of *Flavobacterium collinsii* 983-08^T, *Flavobacterium branchiarum* 57B-2-09^T and *Flavobacterium branchiicola* 59B-3-09^T.
Values are percentages of total CFAs; only CFAs values greater than 1.0% are indicated; tr = trace amount, i.e., < 1.0%. The three strains were cultivated under the same conditions.

Fatty acid	*F. collinsii* 983-08^T	*F. branchiarum* 57B-2-09^T	*F. branchiicola* 59B-3-09^T
$C_{12:1}$	1	tr	tr
$C_{14:0}$ aldehyde	2	tr	tr
iso-$C_{15:0}$ aldehyde	3	4	2
iso-$C_{14:0}$	tr	1	1
$C_{14:0}$	1	tr	tr
iso G-$C_{15:1}$	6	7	5
iso-$C_{15:0}$	29	21	27
anteiso-$C_{15:0}$	1	3	2
$C_{15:1}$ *ω6c*	6	6	8
$C_{15:0}$	7	7	10
iso H-$C_{16:1}$	tr	2	1
iso-$C_{16:0}$	tr	1	1
$C_{16:1}$ *ω7c*	17	13	11
$C_{16:1}$ *ω5c*	1	–	tr
$C_{16:0}$	1	1	1
iso-$C_{15:0}$ 3OH	6	6	6
iso $C_{17:1}$ *ω9c*	3	6	5
$C_{17:1}$ *ω8c*	tr	1	tr
$C_{17:1}$ *ω6c*	2	4	2
iso-$C_{16:0}$ 3OH	1	2	2
$C_{16:0}$ 3OH	3	2	2
iso-$C_{17:0}$ 3OH	3	5	5
Summed feature 2[a]	2	1	2
Unknown fatty acids			
ECL 11.541	3	3	2
ECL 12.555	1	3	1

Summed feature represents a set of more than one CFA that could not be distinguished.
[a] Summed feature 2 comprised iso-$C_{17:1}$ I/DMA $C_{16:0}$.

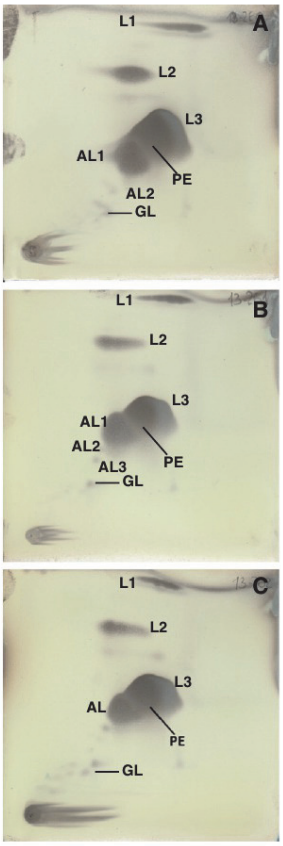

Fig. 2. Total polar lipid profile of strains 983-08^T (A), 57B-2-09^T (B) and 59B-3-09^T (C) after two dimensional thin layer chromatography and staining with molybdatophosphoric acid. PE, phosphatidylethanosamine; AL, unidentified aminolipid; L, unidentified polar lipids; GL, unidentified glycolipid.

profile 1663344. Cells of group A are rods, 0.3 μm wide by 3 μm long. They do not reduce nitrate and nitrite and do not hydrolyze urea, but they degrade tyrosine. They use N-acetyl-glucosamine, maltose and citrate as sole carbon and energy sources but not arabinose and mannitol. They do not exhibit valine arylamidase, β-galactosidase, α-glucosidase, trypsin, β-glucosidase and N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities. Cells of group B are rods, 0.1 μm wide by 2.5 μm long. They do not reduce nitrate and nitrite, do not hydrolyse urea and do not degrade tyrosine. They use N-acetyl-glucosamine, maltose and citrate as sole carbon and energy sources but not arabinose and mannitol. They do not exhibit valine arylamidase and N-acetyl-β-glucosaminidase, trypsin, β-galactosidase, α-glucosidase, β-glucosidase α-mannosidase and α-fucosidase activities. On the other hand, cells of group C are rods, 0.2 μm wide by 2.2 μm long. They reduce nitrate and nitrite, hydrolyse urea and degrade tyrosine. They use N-acetyl-glucosamine, maltose and arabinose as sole carbon and energy sources but not citrate and mannitol. They exhibit N-acetyl-β-glucosaminidase and valine arylamidase activities but activity for trypsin, β-galactosidase, α-glucosidase, β-glucosidase, α-mannosidase and α-fucosidase were not detected. Further characteristics of each group and the phenotypic characteristics that differentiated isolates of groups A, B and C from phylogenetically related species are shown in Table 2. The number of *Flavobacterium* species isolated from fish has increased during recent years, so their identification is necessary in routine bacteriological analysis. Despite the identification of flavobacteria being sometimes difficult as it is based solely in phenotypic criteria (Bernardet and Bowman, 2006, 2011), the characteristics shown in Table 3 could aid their correct identification in clinical microbiology laboratories.

Widely spread microorganisms are usually genetically diverse (Martín et al., 2007). Members of the genus *Flavobacterium* are recovered from a wide range of environments (Bernardet and Bowman, 2006) and therefore this diversity would be expected. After PFGE typing, isolates of the phylogenetic groups A–C exhibited identical or close genetically related profiles. Thus, isolates of group A displayed 4 different restriction profiles (Fig. 3A), and isolates of group B exhibited a unique PFGE profile that was different to those exhibited by the single PFGE profile displayed by the three isolates of group C (Figs. 3B and 3C). These results indicate that isolates of groups B and C represent different single strains. They were obtained from trout from different ponds on the fish farm, which may indicate that groups B and C represent two different clones that are widespread in the fish farm environment.

Most isolates of groups A–C were recovered from gills indicating that they are probably commensal organisms that are able to colonize this tissue. However, two isolates of group A with undistinguishable PFGE profile (Fig. 3A) were recovered from the liver of two different trout, suggesting their ability to infect that species. Although this data is not sufficient to consider this strain as pathogenic for fish, it should be remembered that flavobacteria are considered opportunistic fish pathogens responsible for diseases under different predisposing conditions (Georgiadis et al., 2001; Bernardet and Bowman, 2006).

Table 2
Characteristics that differentiate *Flavobacterium collinsii* 983-08^T, *Flavobacterium branchiarum* 57B-2-09^T and *Flavobacterium branchiicola* 59B-3-09^T from their closely related *Flavobacterium* species according to the phylogenetic tree topology.
Taxa: 1, *Flavobacterium collinsii* 983-08^T; 2, *Flavobacterium branchiarum* 57B-2-09^T; 3, *Flavobacterium branchiicola* 59B-3-09^T; 4, *F. aquidurense* WB-1.1.56^T; 5, *F. araucananum* LM-19-Fp^T; 6, *F. hydatis* DSM 2063^T; 7, *F. granuli* Kw05^T; 8, *F. frigidimaris* KUC-1^T; 9, *F. hibernum* ATCC 51468^T. Data are taken from this study. +, positive reaction; −, negative reaction.

Characteristic	1	2	3	4	5	6	7	8	9
Growth on marine agar	−	−	−	−	−	−	−	+	−
Growth at 30 °C	−	−	−	+	+	+	+	−	−
Degradation of:									
Casein	+	+	+	+	+	+	−	+	+
DNA	−	−	−	−	−	+	−	−	−
L-Tyrosine	+	−	+	+	−	+	−	−	+
Nitrate reduction	−	−	+	−	+	+	−	−	+
Hydrolysis of:									
Urea	−	−	+	+	−	−	−	−	−
Esculin	+	+	+	+	+	+	−	+	+
Assimilation of:									
Arabinose	−	−	+	+	+	−	−	+	+
Mannitol	−	−	−	−	−	−	−	+	−
N-acetyl-glucosamine	+	+	+	+	+	+	+	+	+
Malate	−	−	+	−	−	−	−	−	−
Citrate	+	+	−	−	−	−	−	−	−
Production of:									
Valine arylamidase	−	+	+	+	+	+	+	+	−
Trypsin	−	−	−	−	−	−	+	−	−
β-Galactosidase	−	−	−	−	−	−	+	−	−
α-Glucosidase	−	−	−	−	+	+	−	+	+
β-Glucosidase	−	−	−	−	+	−	−	+	+
N-Acetyl-β-glucosaminidase	−	+	+	−	−	+	+	+	+

4. Conclusion

Overall, the phylogenetic, genotypic and phenotypic results of the present polyphasic study demonstrated that the isolates obtained from rainbow trout represent three novel species of the genus *Flavobacterium*. The names proposed are *Flavobacterium collinsii*, *Flavobacterium branchiarum* and *Flavobacterium branchiicola*. These species have been isolated mainly from trout gills, but some strains would be able to infect this fish species. The description of these new *Flavobacterium* species will facilitate their recognition and identification in the future, improving the knowledge of their distribution and possible association with disease.

4.1. Description of *F. collinsii sp. nov.*

F. collinsii (col.lins'i.i. N.L. gen. masc. n. collinsii of Collins, named after Matthew David Collins, English microbiologist, in recognition to his contributions made to the field of bacterial systematics).

Cells are Gram-negative rods, 0.3 μm wide by 3 μm long, non-endospore-forming, and non-gliding. Strains grow well under aerobic conditions and grow weakly under micro-aerobic conditions. Strains grow at 15–25 °C with optimal growth at approximately 22 °C, while no growth occurs at 30 °C, 37 °C or 42 °C. Growth occurs on trypticase-soy and nutrient agars but not on marine agar after incubation at 25 °C for 72 h. Growth does not occur in the brain heart infusion broth containing 3%, 4.5% or 6.5% NaCl. Colonies are circular, yellow-pigmented, smooth and entire on TGE agar after 72 h of incubation at 25 °C. Colonies are non-haemolytic on Columbia agar after 72 h of incubation at 25 °C. Diffusible flexirubin-type pigments are produced. Congo red is not absorbed by colonies. Catalase and oxidase are produced. Nitrate and nitrite are not reduced. Starch, tyrosine and casein are degraded but gelatine, DNA and agarose are not. A brown pigment is not produced on tyrosine agar. Esculin is hydrolyzed but not urea, lecithin or arginine. Indole and H_2S are not produced. Acid is not produced from D-glucose. Glucose, mannose, N-acetyl-glucosamine, maltose and citrate are used as sole carbon and energy sources but not arabinose, mannitol, gluconate, caprate, adipate, and malate. Activities for alkaline phosphatase, leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase are detected. Esterase C4, valine arylamidase, β-galactosidase, ester lipase C8, lipase C14, α-glucosidase, cystine arylamidase, α-chymotrypsin, trypsin, α-galactosidase, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are not detected. Cells contain menaquinone-6 (MK-6) as the major respiratory quinone. The major cellular fatty acids are iso-$C_{15:0}$, $C_{16:1}$ *ω7c* and $C_{15:0}$.

The type strain, 983-08^T (= CECT 7796^T = CCUG 60109^T), was isolated from the liver of a rainbow trout. The DNA G + C content of this strain is 35.5 mol%.

Table 3
Characteristics that can be useful to differentiate *Flavobacterium* species isolated from fish[a].
Taxa: 1, *F. branchiarum*; 2, *F. branchiicola*; 3, *F. collinsii*; 4, *Flavobacterium onchorynchi*; 5, *Flavobacterium plurextorum*; 6, *F. psychrophilum*; 7, *F. branchiophilum*; 8, *F. columnare*; 9, *F. hydatis*; 10, *F. johnsoniae*; 11, *F. succinicans*; 12, *F. chilense*; 13, *F. araucananum*. Data taken from this study and references Bernardet and Bowman (2006), Kämpfer et al. (2012) and Zamora et al. (2012).

Characteristics	1	2	3	4	5	6	7	8	9	10	11	12	13
Source	Gills	Gills	Viscera	Gills and viscera	Gills and viscera	Skin lesions and viscera	Gills	Skin lesions and viscera	Gills	Skin lesion	Skin lesions	Skin lesions	Skin lesions and viscera
Adsorption of Congo red	−	−	−	−	−	−	−	+	−	−	−	−	−
Flexirubin type pigments	+	+	+	+	+	+	−	+	+	+	−	+	+
Growth on:													
Nutrient agar	+	+	+	+	+	+	−	+	+	+	+	+	+
Tryptic soy agar	+	+	+	+	+	−	−	−	+	+	+	+	+
Temperature range (°C)	15–25	15–25	15–25	15–30	15–30	5–23	5–30	15–37	5–35	10–30	2–< 37	4–30	4–30
Facultative anaerobe	(+)	(+)	(+)	(+)	(+)	−	−	−	+	V	+	−	−
Acid produced from carbohydrates	−	−	−	−	−	−	+	−	+	+	+	+	+
Degradation of:													
Gelatine	−	−	−	−	−	+	+	+	+	+	(+)	+	+
Casein	+	+	+	V	−	+	+	+	+	+	+	+	+
Starch	+	+	+	+	+	−	+	+[b]	+	+	+	+	+
Esculin	+	+	+	+	+	−	−	−	+	+	+	+	+
DNA	−	−	−	−	−	(+)	−	+	+	+	+	nd	nd
Urea	−	+	−	−	−	−	−	−	−	+	−	−	−
Tyrosine	−	+	+	−	+	V	+	−	+	+	−	+	+
Brown diffusible pigment on tyrosine agar	−	−	−	+	−	−	−	V	−	V	−	nd	nd
Production of:													
β-galactosidase	−	−	−	+	−	−	+	−	+	+	+	nd	nd
H2S	−	−	−	−	−	−	−	+	−	−	−	−	+
Nitrate reduction	−	+	−	+	+	−	−	V	+	V	V	nd	nd

[a] Symbols: +, positive reaction; −, negative reaction; V, variable reaction; (+) weak or delayed reaction; nd, no data available.
[b] Degradation of starch by *F. columnare* strains is medium-dependent.

4.2. Description of *F. branchiarum* sp. nov.

F. branchiarum (bran.chi.a'rum. L. gen. pl. n. branchiarum of the gills of fish).

Cells are Gram-negative rods, 0.1 μm wide by 2.5 μm long, non-endospore-forming, and non-gliding. Strains grow well under aerobic conditions and grow weakly under micro-aerobic conditions. Strains grow at 15–25 °C with optimal growth at approximately 20 °C, while no growth occurs at 30 °C, 37 °C or 42 °C. Growth occurs on trypticase-soy and nutrient agars but not on marine agar after incubation at 25 °C for 72 h. Growth does not occur in the brain heart infusion broth containing 3%, 4.5% or 6.5% NaCl. Colonies are circular, yellow-pigmented, smooth and entire on TGE agar after 72 h of incubation at 25 °C. Colonies are non-haemolytic on Columbia agar after 72 h of

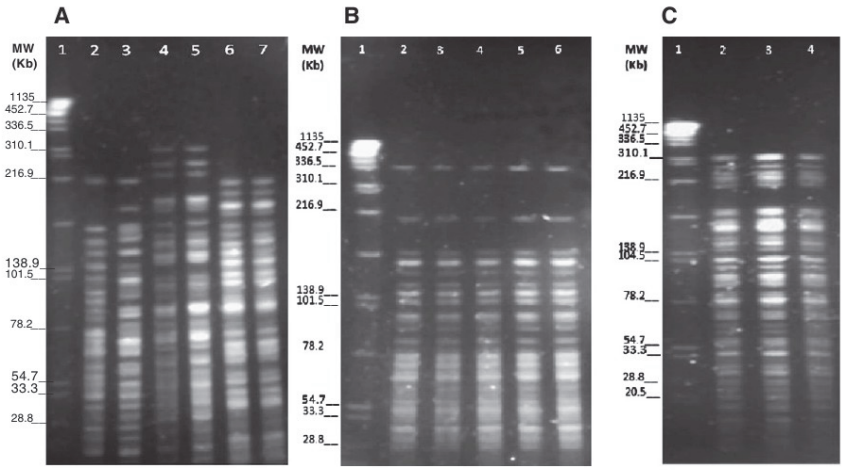

Fig. 3. PFGE patterns generated after *Xho*I-digested chromosomal DNA of *Flavobacterium branchiarum* sp. nov. (Panel A; Lane 1, Molecular weight marker, lines 2 to 7, strains 978B-08, 53B-3-09, 983-08^T, 991-08, 977H-09, 976H-09, respectively), *Flavobacteruium branchiicola* sp. nov. (Panel B; Lane 1, Molecular weight marker; lines 2 to 6, strains 57B-2-09^T, 55B-09, 61B-09, 62B-2-09, 56B-1-09, respectively) and *Flavobacteruium collinsii* sp. nov. (Panel C; Lane 1, Molecular weight marker; lines 2 to 4, strains 59B-3-09^T, 60B-4-09, 58B-1-09, respectively).

incubation at 25 °C. Diffusible flexirubin-type pigments are produced. Congo red is not absorbed by colonies. Catalase and oxidase are produced. Nitrate and nitrite are not reduced. Starch and casein are degraded but tyrosine, gelatine, DNA and agarose are not. A brown pigment is not produced on tyrosine agar. Esculin is hydrolyzed but not urea, lecithin or arginine. Indole and H_2S are not produced. Acid is not produced from D-glucose. Glucose, mannose, N-acetyl-glucosamine, maltose and citrate are used as sole carbon and energy sources but not arabinose, mannitol, gluconate, caprate, adipate, and malate. Activities for alkaline phosphatase, leucine arylamidase, valine arylamidase, N-acetyl-β-glucosaminidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase are detected. Esterase C4, β-galactosidase, ester lipase C8, lipase C14, α-glucosidase, cystine arylamidase, α-chymotrypsin, trypsin, α-galactosidase, β-glucuronidase, β-glucosidase, α-mannosidase and α-fucosidase are not detected. Cells contain menaquinone-6 (MK-6) as the major respiratory quinone. The major cellular fatty acids are iso-$C_{15:0}$ and $C_{16:1}$ *ω7c*.

The type strain, 57B-2-09^T (= CECT 7908^T = CCUG 60095^T), was isolated from the gills of a rainbow trout. The DNA G + C content of this strain is 35.4 mol%.

4.3. Description of *F. branchiicola* sp. nov.

F. branchiicola (bran.chi.i'co.la. L. pl. n. branchiae the gills of fish; L. suff. -cola inhabitant, dweller; N.L. n. branchiicola inhabitant of gills).

Cells are Gram-negative rods, 0.2 μm wide by 2.2 μm long, non-endospore-forming, and non-gliding. Strains grow well under aerobic conditions and grow weakly under micro-aerobic conditions. Strains grow at 15–25 °C with optimal growth at approximately 20 °C, while no growth occurs at 30 °C, 37 °C or 42 °C. Growth occurs on trypticase-soy and nutrient agars but not on marine agar after incubation at 25 °C for 72 h. Growth does not occur in the brain heart infusion broth containing 3%, 4.5% or 6.5% NaCl. Colonies are circular, yellow-pigmented, smooth and entire on TGE agar after 72 h of incubation at 25 °C. Colonies are non-haemolytic on Columbia agar after 72 h of incubation at 25 °C. Diffusible flexirubin-type pigments are produced. Congo red is not absorbed by colonies. Catalase and oxidase are produced. Nitrate and nitrite are reduced. Starch, tyrosine, and casein are degraded but gelatine, DNA and agarose are not. A brown pigment is not produced on tyrosine agar. Esculin and urea are hydrolyzed but not lecithin or arginine. Indole and H_2S are not produced. Acid is not produced from D-glucose. Glucose, arabinose, mannose, N-acetyl-glucosamine, maltose and malate are used as sole carbon and energy sources but not mannitol, gluconate, caprate, adipate, and citrate. Activities for alkaline phosphatase, leucine arylamidase, valine arylamidase, acid phosphatase, N-acetyl-β-glucosaminidase, and naphthol-AS-BI-phosphohydrolase are detected. Esterase C4, β-galactosidase, ester lipase C8, lipase C14, α-glucosidase, cystine arylamidase, α-chymotrypsin, trypsin, α-galactosidase, β-glucuronidase, β-glucosidase, α-mannosidase and α-fucosidase are not detected. Cells contain menaquinone-6 (MK-6) as the major respiratory quinone. The major cellular fatty acids are iso-$C_{15:0}$, $C_{16:1}$ *ω7c* and $C_{15:0}$.

The type strain, 59B-3-09^T (= CECT 7790^T = CCUG 60096^T), was isolated from the gills of a rainbow trout. The DNA G + C content of this strain is 33.6 mol%.

Author contributions

Conceived and designed the experiments: J.F. Fernández-Garayzábal, A.I. Vela, L. Zamora. Performed the experiments: L. Zamora, C. Sánchez-Porro. Obtained specimens: M.A. Palacios. Analyzed the data: E.R.B. Moore, A.I. Vela, A. Ventosa. Wrote the paper: L. Zamora, J.F. Fernández-Garayzábal, A.I. Vela. Critical revision and final approval: J.F. Fernández-Garayzábal, E.R.B. Moore, A. Ventosa.

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5.2 Caracterización taxonómica de microorganismos del género *Chryseobacterium*

El género *Chryseobacterium* fue propuesto por primera vez en el año 1994 por Vandamme y col. como un miembro de la familia *Flavobacteriaceae*, quedando formado inicialmente por seis especies (*C. balustinum*, *C. gleum*, *C. indologenes*, *C. indoltheticum*, *C. meningosepticum*, y *C. scophthalmum*) previamente clasificadas en el género *Flavobacterium*. En la última década el número de especies incluidas en el género ha aumentado considerablemente estando compuesto en la actualidad, octubre de 2014, por 81 especies válidamente publicadas (Euzeby y Parte, 2014a).

Los microorganismos pertenecientes a este género pueden encontrarse en una gran variedad de hábitats que incluyen ambientes naturales como agua, suelo y la rizosfera de diversas plantas; productos alimenticios tales como pescados, carnes, aves de corral y productos lácteos; y entornos industriales como por ejemplo sedimentos contaminados industrialmente y aguas residuales. En medicina humana, algunas especies de *Chryseobacterium* (*C. hominis*, *C. anthropi*, *C. gleum*, *C. treverense*, *C. indologenes* o *C. bernardetii*) se han asociado con diferentes procesos clínicos, si bien los microrganismos de este género son considerados patógenos oportunistas causantes de infecciones nosocomiales principalmente en recién nacidos y pacientes inmunocomprometidos (Hsueh y col., 1997; Chiu y col., 2000; Bernardet y col., 2006; Holmes y col., 2013). En el ámbito veterinario, estos microorganismos no son patógenos relevantes para las especies de animales domésticos. Sin embargo, ha habido un número creciente de infecciones en peces asociadas con diferentes especies de *Chryseobacterium*. Tal es el caso de *C. balustinum*, *C. scophtalmum* y *C. joostei* aisladas de peces enfermos (Harrison, 1929; Mudarris y Austin, 1989; Mudarris y col., 1994; Bernardet y col., 2005; Bernardet y col., 2006). Más recientemente, *C. piscicola* ha sido responsable de casos de mortalidad en trucha arcoíris (*Oncorhynchus mykiss*) y salmón del atlántico (*Salmo salar*) en Chile y Finlandia (Ilardi y col., 2009), *C. arothri* fue aislado de los riñones del pez globo (*Arothron hispidus*) en Hawaii (Campbell y col., 2008) y *C. chaponense* se aisló de lesiones externas, branquias y aleta de salmón del atlántico enfermo (Kämpfer y col., 2011).

En este capítulo se incluyen una serie de trabajos en los que se realiza el estudio taxonómico de una serie de aislados recuperados a partir de

alevines de trucha arcoíris enfermos que presentaban síntomas clínicos compatibles con una infección por *F. psychrophilum*. El aislamiento se realizó a partir de branquias y órganos internos en agar *Anacker and Ordal*, medio comúnmente empleado para el aislamiento de *F. psycrophilum*, en el que crecieron formado colonias de color naranja. Sin embargo, la identificación como *F. psycrophilum* no pudo confirmarse mediante la utilización de una PCR específica para este patógeno (Wiklund y col., 2000).

La primera aproximación a la identificación de estos microorganismos la realizamos mediante la secuenciación del gen que codifica para el 16S ARNr. El análisis de las secuencias de dicho gen permitió la adscripción de los mismos a cuatro grupos filogenéticos (C1-C4) que presentaron porcentajes de similitud en la secuencia del gen 16S ARNr mayores del 99,1% con distintas especies del género *Chryseobacterium*.

Los tres primeros trabajos agrupados en este capítulo abarcan un amplio estudio taxonómico polifásico que incluye la caracterización clásica, bioquímica y fisiológica, así como el análisis quimiotaxonómico y filogenético y estudios de hibridación ADN-ADN y del contenido en G+C del ADN de los aislados de los grupos C1- C3.

Los estudios de hibridación ADN-ADN entre los aislados de los grupos C1-C3 y las especies filogenéticamente más próximas mostraron porcentajes de hibridación que oscilaron entre 2 y 59,4% con las especies filogenéticamente más próximas, valores claramente inferiores al porcentaje del 70% de homología considerado como mínimo para asignar dos taxones a la misma especie (Wayne y col., 1987). El contenido en G+C del ADN varió entre 33,1 y 39,0 mol%, valores que están dentro del rango descrito para el género *Chryseobacterium* (Bernardet y col., 2006; Bernardet y Bowman, 2010). Estos resultados permitieron identificar tres nuevas especies pertenecientes al género *Chryseobacterium* para las que se propusieron los nombres específicos: *Chryseobacterium oncorhynchi* sp. nov. (grupo C1), *Chryseobacterium tructae* sp. nov. (grupo C2) y *Chryseobacterium viscerum* sp. nov. (grupo C3).

Las tres especies presentaron MK-6 como principal quinona respiratoria, el ácido graso mayoritario fue el iso-$C_{15:0}$ y la fosfatidiletanolamina fue el principal lípido polar en las tres especies estudiadas. Estos resultados quimiotaxonómicos confirman la adscripción de las tres nuevas especies al género *Chryseobacterium* basada en los estudios filogenéticos.

Las nuevas especies del género *Chryseobacterium* pueden diferenciarse por pruebas como el crecimiento en medio BHI con 3% de NaCl (observado solamente en la especie *C. tructae*), la utilización de D-manitol como fuente de carbono y energía y la presencia de actividad N-acetil-beta-glucosaminidasa (ambas positivas únicamente para *C. viscerum*), la presencia de las enzimas tripsina y α-glucosidasa en *C. viscerum* y *C. oncorhynchi,* y la ausencia de actividad fosfoamidasa en *C. oncorhynchi.* Igualmente pueden diferenciarse tanto de las especies filogenéticamente más próximas como del resto de especies de *Chryseobacterium* aisladas hasta el momento de peces enfermos.

En el último trabajo recogido en este capítulo se describe la identificación como *Chryseobacterium shigense* de los aislados del grupo C4 mediante el análisis de las secuencias del gen que codifica para 16S ARNr y estudios de caracterización fenotípica. Esta especie fue aislada originalmente de una bebida de ácido láctico y es ésta la primera vez que se aísla a partir de muestras clínicas de peces enfermos.

Con este estudio se incrementa el conocimiento sobre la diversidad de especies del género *Chryseobacterium* relacionadas con infecciones en peces y aunque la identificación de estos microorganismos en el laboratorio es en ocasiones difícil se describen una serie de pruebas que pueden ayudar a la correcta identificación de las distintas especies de este género asociadas a procesos clínicos en peces.

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Chryseobacterium oncorhynchi sp. nov., isolated from rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

Genotypic and phenotypic analyses were performed on five Gram-negative, catalase and oxidase-positive, rod-shaped bacteria isolated from the gill and liver of four rainbow trout. Studies based on comparative 16S rRNA gene sequence analysis showed that the five new isolates shared 99.8–100% sequence similarity and that they belong to the genus *Chryseobacterium*. The nearest phylogenetic neighbours of the strain 701B-08^T were *Chryseobacterium ureilyticum* F-Fue-04IIIaaaa^T (99.1% 16S rRNA gene sequence similarity) and *Chryseobacterium joosteii* LMG 18212^T (98.6%). DNA–DNA hybridization values between the five isolates were 91–99% and ranged from 2 to 53% between strain 701B-08^T and the type strains of phylogenetically closely related species of *Chryseobacterium*. Strain 701B-08^T had a DNA G+C content of 36.3 mol%, the major fatty acids were iso-$C_{15:0}$, iso-$C_{17:1}$ $\omega 9c$, $C_{16:1}$ $\omega 6c$ and iso-$C_{17:0}$ 3-OH and the predominant respiratory quinone was MK-6. The novel isolates were distinguished from related *Chryseobacterium* species by physiological and biochemical tests. The genotypic and phenotypic properties of the isolates from rainbow trout suggest their classification as representatives of a novel species of the genus *Chryseobacterium*, for which the name *Chryseobacterium oncorhynchi* sp. nov. is proposed. The type strain is 701B-08^T (=CECT 7794^T = CCUG 60105^T).

Introduction

The genus *Chryseobacterium* was proposed by Vandamme et al. [35] in the course of subdividing the genus *Flavobacterium*. At the time of writing, *Chryseobacterium* contained 60 species with validly published names [http://www.bacterio.net; 9] and novel taxa are continuously arising within this group; *Chryseobacterium meningosepticum* and *Chryseobacterium miricola* were reclassified as *Elizabethkingia* [19] and *Chryseobacterium arothri* was reclassified as *Chryseobacterium hominis* [17]. Members of the genus *Chryseobacterium* have been isolated from soil and aquatic environments [16,20,38] and are also recovered from food sources [12,15,35]. Some species can also be important as novel sources of bioactive compounds [6]. Moreover, some *Chryseobacterium* species, such as *Chryseobacterium indologenes*, have been considered to be of clinical relevance in human medicine and have been implicated in nosocomial infections, usually in neonates or immuno-compromised patients [4,23]. In veterinary medicine, members of *Chryseobacterium* are not relevant pathogens for domestic animal species, although they have been associated with diseases of freshwater and marine fish [4,11,13,26,27].

In the present study, we report the discovery of a novel species during microbiological diagnoses of different rainbow trout (*Oncorhynchus mykiss*) submitted to the Animal Health Surveillance Centre (VISAVET) of the Universidad Complutense (Madrid, Spain) with a presumptive diagnosis of septicaemia. The taxonomic status of the isolates was determined using a polyphasic approach.

Materials and methods

Isolation, morphological, physiological and biochemical characteristics

Five Gram-negative, rod-shaped bacteria were recovered from the liver (strains 449-08 and 698-2-08) and gills (strains 449B-08, 701B-08^T and 711B-08) of four different trout. The strains were

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Table 1
Cell fatty acid (CFA) profiles of *Chryseobacterium onchorynchi* 701B-08^T and the type strains of related species of genus *Chryseobacterium*: 1, 701B-08^T; 2, *C. ureilyticum* CCUG 52546^T; 3, *C. joostei* CCUG 46665^T; 4, *C. jejuense* CCUG 61058^T; 5, *C. gleum* CCUG 14555^T; 6, *C. oranimense* CCUG 61056^T; 7, *C. indologenes* CCUG 14556^T; 8, *C. artrosphaerae* CCUG 57618^T; 9, *C. shigense* CCUG 61059^T; 10, *C. aquifrigidense* CCUG 61061^T; 11, *C. luteum* CCUG 61057^T; 12, *C. culicis* CCUG 58784^T; 13, *C. lathyri* CCUG 61060^T; 14, *C. vrystaatense* CCUG 50970^T. All strains were cultivated under the same conditions for the CFA analyses. Values are percentages of total CFAs; CFAs representing less than 1% in all strains were omitted. tr, trace amount, i.e., <1%. –, not detected.

Fatty acid	1	2	3	4	5	6	7	8	9	10	11	12	13	14
iso-$C_{13:0}$	tr	1.8	1.6	1.4	–	–	–	tr	1.4	–	1.6	1.3	–	1.6
iso-$C_{15:0}$	33.7	42.5	45.3	38.6	37.2	42.0	37.2	37.6	41.7	37.1	44.7	46.2	39.3	46.8
iso-$C_{15:0}$ 3-OH	3.5	3	3.1	2.9	2.3	3.1	3.0	2.4	3.1	3.1	2.5	2.8	3.6	3.2
anteiso-$C_{15:0}$	tr	–	1.5	–	–	–	–	–	tr	–	5.7	–	–	tr
$C_{16:0}$	1.4	tr	1.5	1.6	1.8	1.4	2.1	1.9	1.2	2.8	–	2.4	1.6	1.3
$C_{16:0}$ 3-OH	tr	tr	–	tr	–	–	–	tr	tr	2.2	–	tr	–	tr
$C_{16:1}$ *ω6c*	16.0	11.7	11.8	16.0	14.8	14.0	12.1	12.4	19.6	19.3	8.5	11.3	18.2	9.4
iso-$C_{17:0}$	1.0	tr	1.1	–	1.6	–	–	1.5	–	–	–	2.0	1.4	tr
iso-$C_{17:1}$ *ω9c*	24.6	23.3	21.6	18.4	23.3	21.5	26.8	24.1	16.0	18.2	21.4	11.5	16.4	14.4
iso-$C_{17:0}$ 3-OH	14.1	11.0	–	14.5	13.1	16.0	13.1	14.1	12.0	13.2	14.1	17.1	15.6	12.9
Summed feature 2[a]	1.9	–	2.6	2.4	2.7	1.9	2.4	1.6	2.1	2.4	–	2.0	1.9	1.9
Unknown fatty acids														
ECL 13.566	tr	3.3	8.5	1.1	1.6	–	3.3	2.2	1.3	1.0	1.7	1.1	2.0	3.2
ECL 16.580	1.1	1.0	1.0	1.1	1.0	–	–	tr	–	–	–	1.4	–	tr

[a] Summed feature represents a set of more than one CFA that could not be distinguished. Summed feature 2 comprised $C_{18:2}$ *ω6,9c* and/or anteiso-$C_{18:0}$.

isolated on tryptone glucose extract agar (Difco) after incubation at 22 °C for 72 h, under aerobic conditions.

For the phenotypic characterization, minimal standards for the description of new taxa in the family *Flavobacteriaceae* were followed [3]; a number of key characteristics were determined, using standard procedures [5,32], i.e., Gram staining, production of catalase and oxidase, and hydrolysis of agar, casein, L-tyrosine, aesculin, DNA, urea, gelatin and starch. Growth in brain heart infusion broth was assessed at 15, 25, 30, 37 and 42 °C, with 3.0, 4.5 and 6.5% added NaCl and under anaerobic (with 4–10% CO_2) and micro-aerobic (with 5–15% O_2 and 5–12% CO_2) conditions, using the GasPak Plus and CampyPak Plus systems (BBL), respectively. Growth was tested on MacConkey (bioMérieux), nutrient (Difco) and trypticase-soy (bioMérieux) agar media. The production of flexirubin-type pigments and extracellular glycans were investigated [3]. The strains were characterized biochemically, using the API 20NE and API Zym systems (bioMérieux), according to the instructions of the manufacturer, except that incubation temperature was 25 °C. The type strains of species *Chryseobacterium ureilyticum* CCUG 52546^T, *Chryseobacterium joostei* CCUG 46665^T, *Chryseobacterium jejuense* CCUG 61058^T, *Chryseobacterium gleum* CCUG 14555^T, *Chryseobacterium oranimense* CCUG 61056^T, *Chryseobacterium indologenes* CCUG 14483^T, *Chryseobacterium artrosphaerae* CCUG 57618^T, *Chryseobacterium shigense* CCUG 61059^T, *Chryseobacterium aquifrigidense* CCUG 61061^T, *Chryseobacterium luteum* CCUG 61057^T, *Chryseobacterium culicis* CCUG 58784^T, *Chryseobacterium lathyri* CCUG 61060^T and *Chryseobacterium vrystaatense* CCUG 50970^T were included as references for the investigation of the phenotypic properties of the trout isolates, using the same laboratory conditions.

Isolate 701B-08^T has been deposited in the Spanish Type Culture collection (CECT) and in the Culture Collection of the University of Gothenburg (CCUG) Sweden, under the accession numbers CECT 7794^T and CCUG 60105^T, respectively.

Fatty acid composition and respiratory quinone analyses

Cellular fatty acid methyl ester (FAME) analyses of strain 701B-08^T and the type strains of the phylogenetically most closely related species (Table 1) were grown aerobically, on Columbia II agar base (BBL 4397596) with 5% horse blood, for 24–48 h, at 37 °C, and their cell fatty acid profiles determined, using gas-chromatography (Hewlett Packard HP 5890) and a standardized protocol, similar to that of the MIDI Sherlock MIS system (http://www.ccug.se/pages/CFA_method_2008.pdf). Cell biomass was removed from the agar medium using a plastic inoculating loop, carefully scraped to avoid including medium in the sample. 50–100 mg of biomass were transferred to glass tubes. The bacterial cells were saponified, the released fatty acids were methylated and the fatty acid methyl esters were extracted. Cell fatty acids (CFAs) were identified and the relative amounts of CFAs in a strain were expressed as percentages of the total fatty acids of that strain.

Respiratory quinones of strain 701B-08^T were extracted from 100 mg of freeze dried cell material using the two stage method described by Tindall [33,34] and further separated by thin layer chromatography on silica gel and further analysed by HPLC by the identification service and Dr. Brian Tindall of the DSMZ (Braunschweig, Germany).

16S rRNA gene sequencing

The 16S rRNA gene sequences of the trout isolates were determined as described previously [36] and subjected to a comparative analysis. A large continuous sequence (approximately 1380 bases) of the 16S rRNA genes of the isolates (449-08, 449B-08, 698-2-08, 701B-08^T and 711B-08) was obtained bidirectionally, using universal primers pA (5′-AGAGTTTGATCCTGGCTCAG; positions 8–27, *Escherichia coli* numbering) and pH* (5′-AAGGAGGTGATCCAGCCGCA; positions 1522–1541, *Escherichia coli* numbering). The calculation of pair-wise 16S rRNA gene sequence similarities and the identifications of phylogenetic neighbours were achieved using the EzTaxon server [http://www.eztaxon.org/; 7]. The 16S rRNA gene sequences of the type strain of all validly published species names of the genus *Chryseobacterium* were retrieved from GenBank and aligned with those of the trout species, using the program SeqTools [30]. Phylogenetic trees were constructed according to three different algorithms: neighbour-joining [31], using the programs SeqTools and TREEVIEW [29,30], maximum-likelihood, using the PHYML software [10]; and maximum-parsimony, using the software package MEGA (Molecular Evolutionary Genetics Analysis) version 4 [22]. Genetic distances for the neighbour-joining and the maximum-likelihood algorithms were calculated by the Kimura two-parameter [21] and close-neighbour-interchange (search level = 2, random additions = 100) was applied in the maximum-parsimony analysis. The stability of the groupings was estimated by bootstrap analysis (1000 replications). The GenBank accession numbers for the 16S rRNA gene sequences of the trout strains determined in this study are shown in Fig. 1.

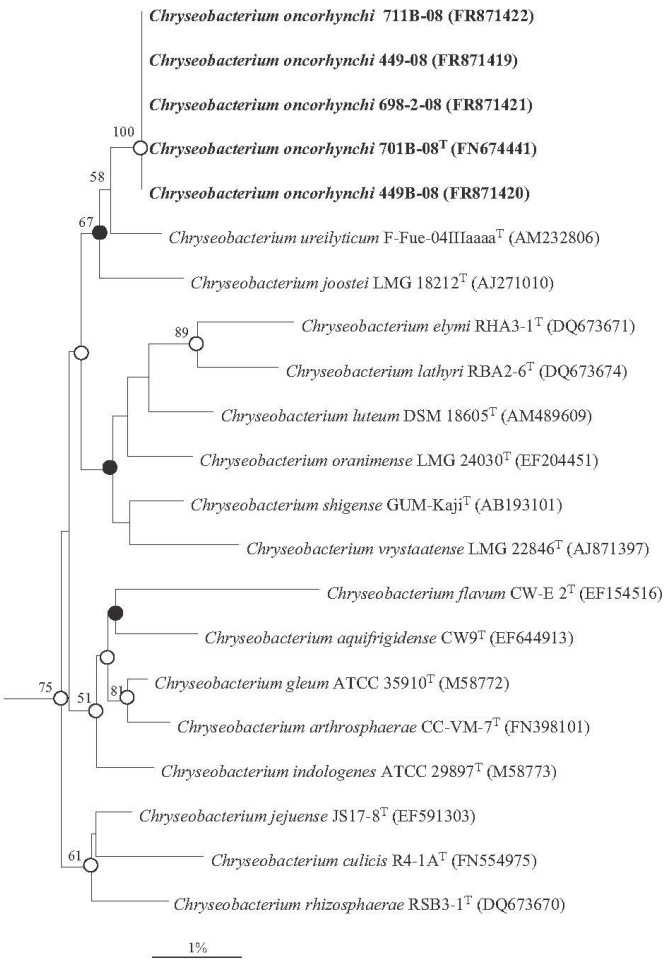

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequence comparison, obtained with the neighbour-joining algorithm, showing the relationships of *Chryseobacterium oncorhynchi* sp. nov. with related taxa. Bootstrap values (expressed as a percentage of 1000 replications) >50% are given at the branching points. Solid circles indicate that the corresponding nodes (groupings) are also obtained on the maximum-likelihood tree. Open circles indicate that the corresponding nodes (groupings) are also obtained on the maximum-likelihood and parsimony trees. *Leeuwenhoekiella marinoflava* ATCC 19326^T (M58770) was used as an outgroup. Bar, 1% sequence divergence.

DNA–DNA hybridization and DNA G + C content

DNA–DNA hybridization experiments were carried out between the five isolates (strains 701B-08^T, 698-2-08, 449-08, 449B-08 and 711B-08) and between strain 701B-08^T and the species with 16S rRNA gene sequence similarities higher than 97.0%: *C. ureilyticum* CCUG 52546^T; *C. joostei* CCUG 46665^T; *C. jejuense* CCUG 61058^T; *C. gleum* CCUG 14555^T; *C. oranimense* CCUG 61056^T; *C. indologenes* CCUG 14483^T; *C. artrosphaerae* CCUG 57618^T; *C. shigense* CCUG 61059^T; *C. aquifrigidense* CCUG 61061^T; *C. luteum* CCUG 61057^T; *C. culicis* CCUG 58784^T; *C. lathyri* CCUG 61060^T; and *C. vrystaatense* CCUG 50970^T. DNA was extracted and purified by the method of Marmur [24]. DNA–DNA hybridization studies were carried out by the membrane method of Johnson [14], described in detail by Arahal et al. [1]. The hybridization experiments were carried out under optimal conditions, at a temperature of 49.5 °C, which is within the limits of validity for the filter method [8]. The percentage of hybridization was calculated as described by Johnson [14]. Three independent determinations were carried out for each experiment and the results reported are mean values.

The G + C contents of the DNAs of the five trout strains were determined from the mid-point values (T_m) of the thermal denaturation profiles [25], using the equation of Owen and Hill [28].

PFGE typing

Strains were grown in trypticase soy agar at 22 °C for 48 h. Cells were suspended with sterile cotton swabs in 1 mL of TE buffer

Table 2
Characteristics that differentiate *Chryseobacterium onchorynchi* sp. nov. from related species of genus *Chryseobacterium*. Strains: 1, 701B-08^T; 2, *C. ureilyticum* CCUG 52546^T; 3, *C. joostei* CCUG 46665^T; 4, *C. jejuense* CCUG 61058^T; 5, *C. gleum* CCUG 14555^T; 6, *C. oranimense* CCUG 61056^T; 7, *C. indologenes* CCUG 14483^T; 8, *C. arthrosphaerae* CCUG 57618^T; 9, *C. shigense* CCUG 61059^T; 10, *C. aquifrigidense* CCUG 61061^T; 11, *C. luteum* CCUG 61057^T; 12, *C. culicis* CCUG 58784^T; 13, *C. lathyri* CCUG 61060^T; 14, *C. vrystaatense* CCUG 50970^T. Data are taken from this study. +, positive reaction; −, negative reaction; (+), weakly positive reaction.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Growth at:														
37 °C	−	−	−	−	+	+	+	+	−	+	−	+	+	−
42 °C	−	−	−	−	+	+	−	+	−	−	−	−	−	−
Tolerance to 3.0% NaCl	−	+	+	+	+	+	+	+	+	−	−	+	−	+
Degradation of:														
Starch	+	+	+	+	+	−	+	+	+	−	−	+	+	+
Gelatin	+	+	+	+	+	+	+	+	+	−	+	+	+	+
Tyrosine	+	+	+	+	−	+	−	+	−	−	−	−	−	+
Brown pigment on tyrosine agar	+	+	+	+	+	−	−	−	−	+	−	+	−	−
Precipitation on egg-yolk medium	−	+	+	(+)	(+)	(+)	(+)	+	(+)	+	+	+	−	−
Production of:														
Indole	+	+	+	+	+	+	+	+	+	−	+	+	−	+
Urease	+	+	+	+	+	−	+	+	−	−	−	+	−	+
β-Galactosidase	−	−	−	−	+	+	+	−	−	−	+	−	−	−
Esterase (C4)	−	−	−	+	−	+	+	+	−	−	−	+	+	+
Esterase lipase (C8)	+	−	+	+	+	+	+	−	+	−	−	−	−	−
Valine arylamidase	+	+	+	+	+	+	+	+	−	+	+	+	+	+
Trypsin	+	−	+	+	−	−	−	+	+	−	+	−	+	−
α-Glucosidase	+	−	−	−	−	−	−	+	−	−	−	−	−	−
β-Glucosidase	−	−	−	−	+	+	−	−	+	−	+	−	−	−
N-Acetyl-β-glucosaminidase	−	−	+	−	−	+	+	+	−	−	−	−	+	−
Naphthol-AS-BI-phophohydrolase	−	+	+	+	+	+	+	+	+	−	+	+	+	+
Assimilation of:														
Arabinose	−	−	−	−	+	+	−	−	−	+	+	+	−	−
Mannose	+	+	+	+	+	+	+	+	+	−	+	+	+	+
Mannitol	−	−	−	−	−	−	−	+	+	−	−	+	−	+
Maltose	+	+	+	+	+	+	+	+	+	−	+	+	+	−
Adipic acid	−	−	−	−	+	+	−	−	−	+	−	−	−	−
Citrate	+	−	−	+	+	+	+	−	−	+	−	+	+	+
Nitrate reduction	−	−	−	−	+	−	−	+	−	+	−	−	−	−

(100 mm Tris, pH 8.0; 100 mm EDTA, pH 8.0) and adjusted to a transmittance of 20%. Agarose plugs were made from a 1:1 mixture of 1% low-melting-point agarose D-1 LOW EEO (Pronadisa) and the cell suspension (200 μl) and 10 μl of proteinase K (20 mg/ml; Roche Applied Science). Plugs were incubated in 5 ml of lysis buffer [1 M Tris–HCl, 0.5 M EDTA, lauroyl sarcosine (10%)] supplemented with 25 μl of proteinase K (1 mg/ml) for 2 h at 54 °C. The cells were washed two times with distillated water and four times with TE 1× buffer for 10 min at 50 °C. Restriction endonuclease digestion was done with the enzyme *Bsp*120I (MBI Fermentas) according to the manufacturer's instructions. The fragments were resolved by PFGE within electrophoresis grade agarose (1%; Pronadisa) in 0.5× TBE (Tris-borate-EDTA) buffer by using a CHEF-DR III System (Bio-Rad). The running conditions were 6 V cm^{-1} at 14 °C. The pulse times ranged from 2.2 to 54 s over 20 h and from 0.1 to 10 s over 16 h. The gels were stained for 30 min with Syber-Safe and photographed under UV light (Gel-Doc, Bio-Rad). Strains were considered different when they differed in at least one band.

Results and discussion

Comparative 16S rRNA gene sequence analysis revealed 99.8–100% sequence similarity between the isolates from diseased trout, demonstrating their high phylogenetic relatedness. Sequence searches showed that the isolates were most closely related to species of the genus *Chryseobacterium*. The 16S rRNA gene sequence of the type strain 701B-08^T exhibited the highest levels of 16S rRNA gene sequence similarity with *C. ureilyticum* and *C. joosteii* (99.1% and 98.6% sequence similarity, respectively). Strain 701B-08^T showed sequence similarities higher than 97.0% with 11 other recognized *Chryseobacterium* species (*C. jejuense, C. gleum, C. oranimens, C. indologenes, C. arthrosphaerae, C. shigense, C. aquifrigidense, C. luteum, C. culicis, C. lathyri* and *C. vrystaatense*). The phylogenetic tree, based on the Neighbour-Joining algorithm (Fig. 1), revealed the clear affiliation of the trout isolates to the genus *Chryseobacterium*, forming a distinct lineage within a subgroup of 16 species, with *C. ureilyticum* and *C. joosteii* as the closest phylogenetic neighbours. The position of this subgroup within the genus *Chryseobacterium* was supported by a bootstrap value of 75%. Similar tree topologies were obtained in phylogenetic trees generated with the maximum-parsimony and maximum-likelihood algorithms used in this study.

The five isolates shared 91–99% genomic DNA–DNA relatedness to each other, demonstrating that they are members of the same species [37]. The strain 701B-08^T showed levels of genomic DNA–DNA relatedness values between 2 and 55% with the type strains of *Chryseobacterium* reference species exhibiting 16S rRNA gene sequence similarities higher than 97.0%. The highest DNA–DNA relatedness value was with *C. gleum* CCUG 14555^T. The strain 701B-08^T showed levels of DNA–DNA relatedness of 53, 52, 39, 34, 42, 20, 36, 34, 20, 20, 26 and 2%, respectively to *C. ureilyticum* CCUG 52546^T, *C. joostei* CCUG 46665^T, *C. jejuense* CCUG 61058^T, *C. oranimense* CCUG 61056^T, *C. indologenes* CCUG 14483^T, *C. artrosphaerae* CCUG 57618^T, *C. shigense* CCUG 61059^T, *C. aquifrigidense* CCUG 61061^T, *C. luteum* CCUG 61057^T, *C. culicis* CCUG 58784^T, *C. lathyri* CCUG 61060^T, and *C. vrystaatense* CCUG 50970^T. These values were significantly lower than the 70% cut-off point recommended for the delineation of bacterial species, confirming that strain 701B-08^T belongs to a distinct genomic species [37].

The DNA G + C content of the five trout strains ranged between 33.1 and 38.5 mol%; the DNA G + C content of strain 701B-08^T was 36.3 mol%, values that were confirmed in three different assays. These DNA G + C content values are consistent with that of the genus *Chryseobacterium* [35].

The CFA profile of strain 701B-08^T was compared with the type strain of the most closely related *Cryseobacterium* species, i.e., as determined by 16S rRNA gene sequence comparative analyses (Table 1). The predominant fatty acids of strain 701B-08^T were iso-$C_{15:0}$ (33.7%), iso-$C_{17:1}$ $\omega 9c$ (24.6%), $C_{16:1}$ $\omega 6c$ (16.0%) and iso-$C_{17:0}$

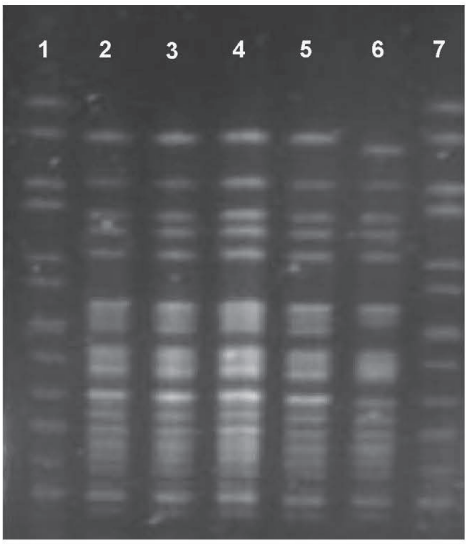

Fig. 2. PFGE patterns generated after *Bsp*120I macrorestriction of *Chryseobacterium oncorhynchi* sp. nov. DNA. Lanes 1 and 7, DNA molecular size marker. Lanes 2–6, strains 449-08, 698-2-08, 449B-08, 701B-08^T and 711B-08, respectively.

3-OH (14.1%) The strain also contained moderate or small amounts of iso-$C_{15:0}$ 3-OH (3.5%), summed feature 2 ($C_{18:2}$ ω6,9*c*/anteiso-$C_{18:0}$) (1.9%), $C_{16:0}$ (1.4%), iso-$C_{17:0}$ (1.0%) and an unknown fatty acid with an equivalent chain length of 16.580 (1.1%). This fatty acid profile is in accordance with those of members of the genus *Cryseobacterium* [35]. The major quinone was MK-6, in accordance with all members of the family *Flavobacteriaceae* [2].

The five isolates exhibited identical biochemical characteristics. A detailed description of the morphological, physiological and biochemical characteristics of the isolates is given in the species description. The physiological and biochemical characteristics that serve to differentiate the new species from related taxa are listed in Table 2. The different responses to growth in the presence of 3.0% NaCl, precipitation on egg-yolk medium, production of the enzymes esterase lipase and α-glucosidase clearly differentiated the trout isolates from *C. ureilyticum* CCUG 52546^T and *C. joostei* CCUG 46665^T, the closet phylogenetic neighbours. Additionally, the trout strains can be differentiated biochemically from other fish pathogens responsible for disease in salmonids, such as *Chryseobacterium piscicola* and *Chryseobacterium chaponense*, by the ability of these species to produce β-glucosidase and their inability to degradate casein and L-tyrosine [13,18]. Moreover, *C. piscicola* grows with 3% NaCl and does not degrade urea and *C. chaponense* grows at 37 °C and does not degrade gelatin and starch.

Visual comparison of the restriction endonuclease digestion profiles generated by PFGE, revealed 2 different profiles represented by isolates 449-08, 698-2-08, 449B-08, 701B-08^T (Fig. 2, lanes 2–5) and 711B-08 (Fig. 2, lane 6) among the trout isolates.

Overall, the results of the present study show that the new isolates from rainbow trout constitute a distinct branch within the genus *Chryseobacterium* (Fig. 1). Given the phenotypic differences and based on genotypic criteria it is clear that the new isolates merit classification as a new species within the genus *Chryseobacterium*, for which the name *Chryseobacterium oncorhynchi* sp. nov. is proposed. The strain 701B-08^T (=CECT 7794^T = CCUG 6010^T) is designated as the type strain for the species.

Description of *Chryseobacterium oncorhynchi* sp. nov.

Chryseobacterium oncorhynchi (on.co.rhyn'chi. N.L. gen. n. oncorhynchi of *Oncorhynchus*, named after the rainbow trout, *Oncorhynchus mykiss*, from which the type strain was isolated).

Cells are Gram-negative rods, 0.7 μm wide by 1.7–2.0 μm long, non-endospore-forming, and non-gliding. Grows well under aerobic conditions, and grows weakly under microaerobic conditions. Grows at 15–30 °C with optimal growth at approximately 25 °C while no growth occurs at 37 °C. Growth occurs on trypticase-soy and nutrient agars but not on MacConkey agar after incubation at 25 °C for 72 h. Growth does not occur in brain heart infusion broth containing 3.0%, 4.5% and 6.5% NaCl. Colonies are circular, pale orange-pigmented, smooth, with entire margins on TGE agar after 72 h of incubation at 25 °C. Colonies are haemolytic on blood agar after 72 h of incubation at 25 °C. Diffusible flexirubin-type pigments are produced. Congo red is not absorbed by colonies. Catalase and oxidase are produced. Nitrate and nitrite are not reduced. Starch, tyrosine, gelatin and casein are hydrolyzed but agar is not. A brown pigment is produced on tyrosine agar. Aesculin, urea and Tween 80 are hydrolyzed but not arginine, lecitin and DNA. Indole is produced but not H_2S. β-Galactosidase activity is not detected. Glucose, mannose and maltose are used as sole carbon and energy source but not citrate, arabinose, N-acetyl-glucosamine, mannitol, gluconate, caprate, adipate, and malate. Acid is not produced from D-glucose. Activities for alkaline phosphatase, ester lipase C8, naphthol-AS-BI-phophohydrolase, leucine arylamidase, α-glucosidase, acid phosphatase, trypsin and valine arylamidase are detected. Esterase C-4, lipase C14, cystine arylamidase, α-chymotrypsin, α-galactosidase, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase, are not detected. Cells contain menaquinone-6 (MK-6) as the major respiratory quinone. The major cell fatty acids are iso-$C_{15:0}$, iso-$C_{17:1}$ ω9*c*, $C_{16:1}$ ω6*c* and iso-$C_{17:0}$ 3-OH. The DNA G + C content ranged between 33.1 and 38.5 mol%.

The type strain, 701B-08^T (=CECT 7794^T = CCUG 60105^T), was isolated from the gill of a rainbow trout. The DNA G + C content of this strain is 36.3 mol%.

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Chryseobacterium tructae sp. nov., isolated from rainbow trout (*Oncorhynchus mykiss*)

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Oncorhynchus mykiss

ABSTRACT

Three pale-orange bacteria (strains 1083-08, 1084-08^T and 1095B-08) were isolated from diseased rainbow trout. The isolates were Gram-staining-negative, catalase- and oxidase-positive, rod-shaped cells. Analyses of their 16S rRNA gene sequences confirmed their adscription to the genus *Chryseobacterium*. The three isolates shared 100% 16S rRNA gene sequence similarity and 98.5% similarity with *Chryseobacterium indologenes* CCUG 14556^T, being the closest phylogenetically related species. Genomic DNA–DNA hybridization similarity values between the three isolates were 94–100% and 2–39% between strain 1084-08^T and the type strains of other related *Chryseobacterium* species, confirming that the isolates represent a novel species within the genus *Chryseobacterium*. The DNA G+C content of the species was 33.6–36.1 mol%. The predominant respiratory quinone of strain 1084-08^T was MK-6 and the major fatty acids were iso-$C_{15:0}$, iso-$C_{17:1}$ ω9c, iso-$C_{17:0}$ 3-OH and $C_{16:1}$ ω6c. The isolates were distinguished from related *Chryseobacterium* species by a number of phenotypic properties. Based on the phenotypic, genotypic and phylogenetic findings, it is proposed that the new isolates from rainbow trout be classified as a new species of the genus *Chryseobacterium*, with the name of *Chryseobacterium tructae* sp. nov. The type strain is 1084-08^T (=CECT 7798^T = CCUG 60111^T).

Introduction

The genus *Chryseobacterium* is a member of the family *Flavobacteriaceae*, phylum *Bacteroidetes*, and represents one of the genera with a rapidly increasing number of species. At the time of writing, the genus *Chryseobacterium* comprised 59 described and validly published species [11]. Members of this genus have been isolated from a variety of environmental sources, including soil, water, sludge, plants, food products such as fish, meat, poultry, milk and lactic acid beverages, and human clinical specimens [5,6]. The significance of *Chryseobacterium* in veterinary medicine is primarily limited to some species, such as *C. balustinum*, *C. scophtalmum*, *C. arothri* (*currently C. hominis*) and *C. joostei*, that have been isolated from diseased fish [4,8,13,14,21,22]. Furthermore, three new species associated with fish disease have been described recently. *C. piscicola* was isolated from external lesions of diseased farmed Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) [14], *C. chaponense* from external lesions, gills and fins of diseased Atlantic salmon [16] and *C. oncorhynchi* from gills and kidney of diseased rainbow trout [34].

In this article, we report the phenotypic, genotypic and phylogenetic characterization of three novel *Chryseobacterium*-like strains isolated from trout. Based on the presented findings, a new species of the genus *Chryseobacterium*, *Chryseobacterium tructae* sp. nov., is proposed.

Materials and methods

Isolation of bacteria

During the routine microbiological diagnosis from different clinical specimens of rainbown trout (*O. mykiss*) submitted to the Animal Health Surveillance Centre (VISAVET) of the Universidad Complutense (Madrid, Spain), three novel Gram-negative, rod-shaped bacteria were recovered from liver, strains 1083-08 (CCUG 60110) and 1084-08^T (CCUG 60111^T), and gills (strain 1095B-08)

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of three different trout with a presumptive diagnosis of septicemia. The isolates were recovered on the same date, from two different ponds (1083-08 and 1084-08 from one pond and 1095B-08 from another pond) in the same fish farm. The strains were isolated on tryptone glucose extract agar (TGE; Difco) after incubation at 25 °C for 72 h under aerobic conditions.

16S rRNA gene sequence determinations and analyses

In order to establish the phylogenetic allocation of the bacteria, the 16S rRNA gene sequences of the three isolates were determined, as described previously [34], and subjected to a comparative analysis. A large continuous sequence (approximately 1400 bases) of the 16S rRNA gene of the three isolates was obtained in both directions, using universal amplification primers: pA (5′-AGAGTTTGATCCTGGCTCAG, positions 8–27, *Escherichia coli* numbering); and pH* (5′-AAGGAGGTGATCCAGCCGCA, positions 1541–1522, *E. coli* numbering). The identifications of the phylogenetic neighbors and calculations of pair-wise 16S rRNA gene sequence similarities were achieved, using the EzTaxon server (http://www.eztaxon.org/) [9]. The 16S rRNA gene sequences of the type strains of all species with valid names of the genus *Chryseobacterium* were retrieved from GenBank and aligned with the newly determined sequences using the program SeqTools [24]. Phylogenetic trees were constructed according to three different algorithms: neighbor-joining [26], using the programs SeqTools and TREEVIEW [23,24]; maximum-likelihood, using the PHYML software [12]; and maximum-parsimony, using the software package MEGA (Molecular Evolutionary Genetics Analysis) version 4 [18]. Genetic distances for the neighbor-joining and the maximum-likelihood algorithms were calculated by the Kimura two-parameter [17] and close-neighbor-interchange (search level = 2, random additions = 100) was applied in the maximum-parsimony analysis. The stability of the groupings was estimated by bootstrap analysis (1000 replications).

Genomic DNA–DNA similarity determinations

Genomic DNA–DNA hybridizations were carried out between the three isolates (strains 1084-08^T, 1083-08 and 1095B-08) and between strain 1084-08^T and the type strains of all species with 16S rRNA gene sequence similarities greater than 97.0%. DNA was extracted and purified by the method of Marmur [19]. Hybridization studies were carried out, using the membrane method of Johnson [15], described in detail by Arahal et al. [1]. The hybridization experiments were carried out under optimal conditions, at a temperature of 44 °C, which is within the limits of validity for the membrane method [10]. The percentages of hybridization similarities were calculated as described by Johnson [15]. Three independent determinations were carried out for each experiment and the results reported as mean values.

DNA G+C content determination

The G+C contents of the genomic DNA of a representative isolate (strain 1084-08^T) and the other two isolates were determined from the mid-point value (T_m) of the thermal denaturation profile [20], obtained with a Perkin-Elmer UV-Vis Lambda 20 spectrophotometer at 260 nm.

Fatty acid composition and respiratory quinone determinations

Respiratory quinones of strain 1084-08^T were extracted from 100 mg of freeze-dried cell material, using the two stage method described by Tindall [29,30], and further separated by thin layer chromatography on silica gel and analyzed, using HPLC, by the identification service of the DSMZ (Braunschweig, Germany).

The cell fatty acid–fatty acid methyl ester (CFA–FAME) analyses of strain 1084-08^T was done with cultures on Columbia II agar base (BBL 4397596) with 5% horse blood, at 30 °C for 30–48 h, under aerobic conditions. The CFA–FAME profile was determined, using gas chromatography (Hewlett Packard HP 5890) and a standardized protocol similar to that of the MIDI Sherlock MIS system [27], described previously [34]. CFAs were identified and the relative amounts were expressed as percentages of the total fatty acids of the strain.

Physiological and biochemical characterization

The minimal standards for the description of new taxa in the family *Flavobacteriaceae* [3] were followed for the phenotypic characterization of the isolates. Gram-staining was performed as described by Smibert and Krieg [28]. Oxidase activity was determined by monitoring the oxidation of tetramethyl-*p*-phenylenediamine on filter paper and catalase activity was determined, using 3% H_2O_2 solution [28]. Hydrolysis of Tween 80 (1%, v/v), L-tyrosine (0.5%, w/v), lecithin (5%, w/v) [28], esculin (0.01% esculin and 0.05% ferric citrate, w/v), gelatin (4%, w/v), starch (0.2%, w/v), and casein [50% skimmed milk (Difco), v/v] were tested using nutrient agar [28,31]. DNase test agar (Difco) was used for the DNase assay. Hydrolysis of urea (1%, w/v) was tested as described by Bowman et al. [7]. Growth in brain heart infusion broth was assessed at 15, 25, 30, 37 and 42 °C, with 3.0, 4.5 and 6.5% added NaCl, and under anaerobic (with 4–10% CO_2) and micro-aerobic (with 5–15% O_2 and 5–12% CO_2) conditions, using GasPak Plus and CampyPak Plus systems (BBL), respectively. Growth was tested on MacConkey (bioMérieux), nutrient (Difco) and trypticase-soy (bioMérieux) agar plates. The presence of gliding motility, using the hanging drop technique, the production of flexirubin-type pigments and extracellular glycans were assessed, using the KOH and Congo red tests, respectively [3]. The strains were further biochemically characterized using the API 20NE and API Zym systems (bioMérieux) according to the manufacturer's instructions, except that incubation temperature was 25 °C. The type strains of species *C. oncorhynchi* 701-08^T, *C. indologenes* CCUG 14556^T, *C. ureilyticum* CCUG 52546^T, *C. gleum* CCUG 14555^T, *C. arthrosphaerae* CCUG 57618^T, *C. jejuense* CCUG 61058^T, *C. hominis* CCUG 52711^T, *C. shigense* CCUG 61059^T, *C. aquifrigidense* CCUG 61061^T and *C. joostei* CCUG 46665^T were included as references for the investigation of the phenotypic properties of the trout isolates under the same laboratory conditions.

PFGE typing

The 3 isolates from diseased trout were characterized by pulsed-field gel electrophoresis (PFGE) profiling of their genomic DNAs, according to the specifications of Zamora et al. [34], with the exception that the restriction enzymes were *Xba*I and *Bcu*I (MBI Fermentans), and the pulse times ranged from 0.1 to 15 s for a period of 30 h. Similarities between restriction endonuclease digestion profiles were based on visual comparisons of the band patterns of isolates run in the same gel. Strains differing in at least one band were considered different.

Results and discussion

Comparative 16S rRNA gene sequence analysis revealed 100% similarity between the sequences of the three isolates from diseased trout, suggesting an identical or close genealogical identity. Sequence searches showed that the isolates were most closely related to members of the genus *Chryseobacterium*. The 16S rRNA

gene sequence of the type strain 1084-08^T exhibited the highest levels of similarity with that of *C. indologenes* CCUG 14556^T (98.5% sequence similarity). The sequence of strain 1084-08^T possessed sequence similarities greater than 97.0% with nine other *Chryseobacterium* species: *C. ureilyticum* CCUG 52546^T (98.0%), *C. gleum* CCUG 14555^T (97.9%); *C. onchorynchis* 707-08^T (97.8%); *C. arthrosphaerae* CCUG 57618^T (97.7%); *C. jejuense* CCUG 61058^T (97.7%); *C. hominis* CCUG 52711^T (97.6%); *C. shigense* CCUG 61059^T (97.2%); *C. aquifrigidense* CCUG 61061^T (97.1%); and *C. joostei* CCUG 46665^T (97.1%).

The reconstructed phylogenetic tree, based on the neighbor-joining algorithm (Fig. 1), as well as two other methods (not shown), revealed a clear affiliation of the trout isolates to the genus *Chryseobacterium*. It is evident from the phylogenetic tree (Fig. 1), that the isolates form a distinct sub-lineage, clustering with a sub-group of 16 species (including the type species of the genus, *C. gleum*), with *C. indologenes* the closest phylogenetic neighbor. This close relatedness between the trout isolates and *C. indologenes* was supported by a bootstrap value of 85%. The branching position of the three strains within this clade was stable, according to the three tree-making algorithms used in this study. The GenBank accession numbers for the 16S rRNA gene sequences of trout strains sequenced in this study are shown in Fig. 1.

The three strains shared 94–100% genomic DNA–DNA similarities to each other, confirming that they are members of the same species [33]. Strain 1084-08^T showed levels of genomic DNA similarity between 2 and 47% with the type strains of *Chryseobacterium* species exhibiting 16S rRNA gene sequence similarities greater than 97.0%: *C. ureilyticum* CCUG 52546^T (39%), *C. oncorhynchi* 701-08^T (45%); *C. joostei* CCUG 46665^T (16%); *C. jejuense* CCUG 61058^T (47%); *C. gleum* CCUG 14555^T (35%); *C. indologenes* CCUG 14483^T (34%); *C. arthrosphaerae* CCUG 57618^T (28%); *C. shigense* CCUG 61059^T (2%); *C. aquifrigidense* CCUG 61061^T (19%); and *C. hominis* CCUG 52711^T (30%). These data clearly confirmed that the new isolates belong to a distinct genomic species [25,33].

The G+C content of strain 1084-08^T was determined to be 37.4 mol%. DNA G+C values of strains 1083-08 and 1095-08 were 33.6 and 34.0 mol%, respectively. These DNA G+C values are consistent with those of species of the genus *Chryseobacterium* [5,6,32].

Adscription of the trout isolates to the genus *Chryseobacterium* was corroborated by CFA analysis. The predominant cell fatty acids of strain 1084-08^T were iso-$C_{15:0}$ (38.5%), iso-$C_{17:1}$ $\omega 9c$ (23.4%), iso-$C_{17:0}$ 3-OH (14.7%) and $C_{16:1}$ $\omega 6c$ (13.1%). This fatty acid profile is in accordance with those of members of the genus *Chryseobacterium* [4,6,32]. The major quinone was MK-6, in accordance with all members of the family *Flavobacteriaceae* [2].

The three isolates exhibited identical physiological and biochemical characteristics profiles. A detailed description of the morphological, physiological and biochemical characteristics of the isolates is given in the species description. The phenotypic characteristics that differentiated the trout isolates from related phylogenetic species are shown in Table 1. The isolates were readily differentiated from *C. indologenes*, the closet neighbor on the basis of 16S rRNA gene sequence similarity, by the ability of the new species to grow at 5 °C. Additionally, *C. indologenes* grows at 37 °C, produces β-galactosidase, esterase C4 and *N*-acetyl-β-glucosaminidase but does not produce gelatinase; the new strains showed the opposite results for these tests. Moreover, the trout strains can be differentiated from other species isolated from diseased fish, such as *C. piscicola*, *C. chaponense* or *C. oncorhynchi*. Thus, *C. piscicola and C. chaponense* produce β-glucosidase but are not able to degrade starch or casein. Moreover, *C. piscicola* does not degrade urea and does not produce indole; *C. chaponense* grows at 37 °C and does not degrade gelatin. *C. oncorhynchi*, in addition to characteristics shown in Table 1, is hemolytic [14,16,34].

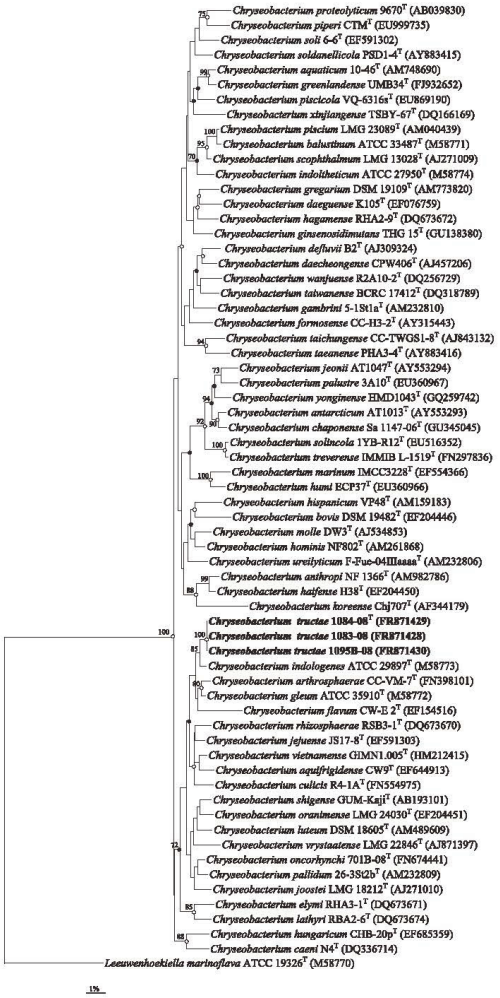

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequence comparisons, obtained with the neighbor-joining algorithm showing the relationships of *Chryseobacterium tructae* sp. nov. with related species. Bootstrap values (expressed as a percentage of 1000 replications) >70% are included, at the branching points. Solid circles indicate that the corresponding nodes (groupings) are also obtained on the maximum-likelihood tree. Open circles indicate that the corresponding nodes (groupings) are also obtained on the maximum-likelihood and parsimony trees. *Leeuwenhoekiella marinoflava* ATCC 19326^T (M58770) was used as an outgroup. Bar, 1% sequence divergence.

After PFGE typing, the three isolates displayed undistinguishable restriction profiles with both enzymes (data not shown), suggesting a clonal relationship that could be related with the fact that they were recovered during the same clinical episode in the same fish farm. The virulence of the new isolates has not been determined yet by challenge experiments.

Overall, the results of the present polyphasic study demonstrate that the characteristics of the new isolates from diseased rainbow

Table 1
Characteristics that differentiate *Chryseobacterium tructae* sp. nov. from related species of the genus *Chryseobacterium*. Strains: 1, 1084-08^T; 2, *C. indologenes* CCUG 14556^T; 3, *C. ureilyticum* CCUG 52546^T; 4, *C. gleum* CCUG 14555^T; 5, *C. arthrosphaerae* CCUG 57618^T; 6, *C. jejuense* CCUG 61058^T; 7, *C. hominis* CCUG 52711^T; 8, *C. shigense* CCUG 61059^T; 9, *C. aquifrigidense* CCUG 61061^T; 10, *C. joostei* CCUG 46665^T; 11, *C. oncorhynchi* 701B-08^T. All data are from this study. +, positive reaction; –, negative reaction.

Characteristics	1	2	3	4	5	6	7	8	9	10	11
Growth on/at:											
Cetrimide agar	+	+	+	–	+	+	–	+	+	+	+
5 °C	+	–	–	–	–	+	–	+	–	+	+
37 °C	–	+	–	+	+	–	+	–	+	–	–
42 °C	–	–	–	+	+	–	–	–	–	–	–
Tolerance to 3.0% NaCl	+	+	+	+	+	+	–	+	–	+	–
Precipitation on egg-yolk medium	+	–	+	+	–	+	+	–	+	+	–
Degradation of:											
Tween 80	+	+	+	+	+	+	–	+	+	+	+
Casein	+	+	+	+	+	+	–	+	+	+	+
Starch	+	+	+	+	+	+	–	+	–	+	+
Gelatin	+	–	+	–	+	+	–	–	–	+	+
Nitrate reduction	–	–	–	+	+	–	+	–	+	–	–
Production of:											
Indole	+	+	+	+	+	+	+	+	–	+	+
Urease	+	+	+	+	+	+	–	–	–	+	+
β-Galactosidase	–	+	–	+	–	–	–	–	–	–	–
Esterase (C4)	–	+	–	–	+	+	–	–	–	–	–
Esterase lipase (C8)	+	+	–	+	–	+	+	+	–	+	+
Valine arylamidase	+	+	+	+	+	+	+	–	+	+	+
Trypsin	–	–	–	–	+	+	–	+	–	+	+
α-Glucosidase	–	–	–	–	+	–	–	–	–	–	+
β-Glucosidase	–	–	–	+	–	–	–	+	–	–	–
N-acetyl-β-glucosaminidase	–	+	–	–	+	–	–	–	–	+	–
Assimilation of:											
Arabinose	–	–	–	+	–	–	–	–	+	–	–
Mannose	+	+	+	+	+	+	+	+	–	+	+
Mannitol	–	–	–	–	+	–	–	+	–	–	–
Maltose	+	+	+	+	+	+	+	+	–	+	+
Adipic acid	–	–	–	+	–	–	–	–	+	–	–
Citrate	+	+	–	+	–	+	–	–	+	–	+

trout match those described for the genus *Chryseobacterium* and should be assigned to this genus. The phylogenetic, genotypic and phenotypic differences clearly demonstrate that the new isolates merit classification as a new species, for which the name *C. tructae* sp. nov. is proposed.

Description of *C. tructae* sp. nov.

C. tructae (truc'ta.e L. gen. n. tructae, of a trout, from which the type strain was isolated).

Cells are Gram-negative rods, 0.7 μm wide and 2.6 μm long, non-endospore-forming, and non-gliding. Strains grow well under aerobic conditions and grow weakly under micro-aerobic conditions. Strains grow at 5–30 °C with optimal growth at approximately 25 °C, while no growth occurs at 37 °C or higher temperatures. Growth occurs on trypticase-soy and nutrient agars but not on MacConkey agar after incubation at 25 °C for 72 h. Growth occurs in brain heart infusion broth containing 3% NaCl, but not in 4.5 and 6.5% NaCl. Colonies are circular, pale orange-pigmented, smooth and entire on TGE agar after 72 h incubation at 25 °C. Colonies are non-hemolytic on Columbia agar after 72 h incubation at 25 °C. Diffusible flexirubin-type pigments are produced. Congo red is not absorbed by colonies. Catalase and oxidase are produced. Nitrate and nitrite are not reduced. Starch, tyrosine, gelatin and casein are degraded but agarose is not. A brown pigment is produced on tyrosine agar. Aesculin, lecithin, urea and Tween 80 are hydrolyzed but not arginine and DNA. Indole is produced but not H_2S. Activity for β-galactosidase is not detected. Acid is not produced from D-glucose. Glucose, mannose, maltose and citrate are used as sole carbon and energy sources but not arabinose, N-acetyl-glucosamine, mannitol, gluconate, caprate, adipate, and malate. Activities for alkaline phosphatase, ester lipase C8, leucine arylamidase, naphthol-AS-BI-phophohydrolase, acid phosphatase and valine arylamidase are detected. Esterase C4, lipase C14, α-glucosidase, cystine arylamidase, α-chymotrypsin, trypsin, α-galactosidase, β-glucuronidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are not detected. Cells contain menaquinone-6 (MK-6) as the major respiratory quinone. The major cellular fatty acids are iso-$C_{15:0}$ (38.5%), iso-$C_{17:1}$ *ω9c* (23.4%), iso-$C_{17:0}$ 3-OH (14.7%) and $C_{16:1}$ *ω6c* (13.1%). The DNA G+C content of the species is 33.6–37.4 mol%.

The type strain, 1084-08^T (=CECT 7798^T = CCUG 60111^T), was isolated from the liver of a rainbow trout. The DNA G+C content of this strain is 37.4 mol%.

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Chryseobacterium viscerum sp. nov., isolated from diseased fish

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A taxonomic study was carried out on five Gram-staining-negative, catalase- and oxidase-positive, rod-shaped bacteria isolated from the gills and livers of five diseased rainbow trout. The five novel isolates were designated strains 687B-08^T, 445-08, 452-08, 453B-08 and 967B-08. In phylogenetic analyses based on 16S rRNA gene sequences, the five novel strains appeared almost identical (99.0–100 % sequence similarity) and to belong to the genus *Chryseobacterium*. Strain 687B-08^T (the strain selected to represent the five novel isolates) was found to be most closely related to *Chryseobacterium oncorhynchi* 701B-08^T (98.9 % sequence similarity), *Chryseobacterium ureilyticum* F-Fue-04IIaaaa^T (98.6 %), *Chryseobacterium indologenes* ATCC 29897^T (98.3 %), *Chryseobacterium jejuense* JS17-8^T (98.1 %) and *Chryseobacterium gleum* ATCC 35910^T (98.1 %). In DNA–DNA hybridizations, DNA–DNA relatedness values of 99–100 % were recorded between the five novel strains. Lower DNA–DNA relatedness values (21–57 %) were recorded between strain 687B-08^T and *C. oncorhynchi* 701B-08^T, *C. ureilyticum* F-Fue-04IIaaaa^T and the type strains of other closely related, established species of the genus *Chryseobacterium*. The predominant respiratory quinone of strain 687B-08^T was MK-6 and the major cellular fatty acids were iso-$C_{15:0}$, iso-$C_{17:1}\omega9c$, iso-$C_{17:0}$ 3-OH and $C_{16:1}\omega6c$. The G+C content of the genomic DNA of strain 687B-08^T was 38.6 mol%. Based on the phenotypic and genotypic evidence, the five novel strains isolated from rainbow trout represent a single, novel species of the genus *Chryseobacterium*, for which the name *Chryseobacterium viscerum* sp. nov. is proposed. The type strain is 687B-08^T (=CECT 7793^T =CCUG 60103^T).

At the time of writing, the genus *Chryseobacterium*, which has recently undergone significant expansion as a result of improvements in phenotypic and molecular identification methods, comprised 58 species with validly published names (Bernardet *et al.*, 2011; http://www.bacterio.cict.fr). This genus, a member of the family *Flavobacteriaceae*, was described formally by Vandamme *et al.* (1994) to accommodate six species formerly classified within the genus *Flavobacterium*. The type species of the genus is *Chryseobacterium gleum*. Members of the genus *Chryseobacterium* can be found in a wide variety of environments, including water, soil, raw milk, raw chicken and clinical samples (Yamaguchi & Yokoe, 2000; Bernardet *et al.*, 2002, 2005, 2006; Hugo *et al.*, 2003; Kim *et al.*, 2006; de Beer *et al.*, 2005, 2006; Quan *et al.*, 2007). Several *Chryseobacterium* species, such as *Chryseobacterium balustinum*, *Chryseobacterium scophthalmum*, *Chryseobacterium joostei* and, more recently, *Chryseobacterium piscicola*, *Chryseobacterium arothri* (a heterotypic synonym of *Chryseobacterium hominis*; Kämpfer *et al.*, 2009), *Chryseobacterium chaponense* and *Chryseobacterium oncorhynchi*, have been associated with infections in fish (Harrison, 1929;

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 687B-08^T, 445-08, 452-08, 453B-08 and 967B-08 are FR871426, FR871423, FR871424, FR871425 and FR871427, respectively.

Mudarris & Austin, 1989; Mudarris *et al.*, 1994; Bernardet *et al.*, 2005; Campbell *et al.*, 2008; Ilardi *et al.*, 2009; Kämpfer *et al.*, 2011; Zamora *et al.*, 2012). In this study, the taxonomic positions of five *Chryseobacterium*-like organisms recovered from five different specimens of trout were determined, using phenotypic, chemotaxonomic and molecular genetic methods.

In 2008, during routine microbiological diagnoses at the Animal Health Surveillance Centre (VISAVET) of the Universidad Complutense (Madrid, Spain), five novel isolates of Gram-staining-negative rod-shaped bacteria were obtained from the liver (strains 445-08 and 452-08) or gills (strains 453B-08, 687B-08^T and 967B-08) of five different rainbow trout (*Oncorhynchus mykiss*). The five fish, which were thought to have suffered from septicaemia, came from a single fish farm in central Spain. The five novel strains, from trout samples that had been held at −40 °C until they could be processed, were isolated on tryptone-glucose extract (TGE) agar (Difco) after incubation at 22 °C for 72 h under aerobic conditions. No attempt has yet been made to test the virulence of any of the novel strains (e.g. by experimental infection of trout).

The 16S rRNA gene sequences of all five novel strains were determined as described previously (Vela *et al.*, 2005). A continuous fragment (of about 1377 nt) of the 16S rRNA gene of each strain was obtained bidirectionally, using the universal primers pA (5′-AGAGTTTGATCCTGGCTCAG-3′; representing positions 8–27 in the *E. coli* numbering system) and pH* (5′-AAGGAGGTGATCCAGCCGCA-3′; representing positions 1541–1522). The EzTaxon server (http://www.eztaxon-e.ezbiocloud.net/; Kim *et al.*, 2012) was then used to identify phylogenetic neighbours and evaluate sequence similarities between pairs of strains. In this phylogenetic analysis, the five novel strains appeared almost identical, with 16S rRNA gene sequence similarities of 99.0–100 %, and to be most closely related to members of the genus *Chryseobacterium*. Strain 687B-08^T (selected to represent the five novel strains) appeared to be most closely related to *C. oncorhynchi* 701B-08^T (98.9 % sequence similarity) and *Chryseobacterium ureilyticum* F-Fue-04IIIaaaa^T (98.6 %). Sequence similarities higher than 97 % were observed between strain 687B-08^T and type strains of 12 other established species in the genus *Chryseobacterium*: *Chryseobacterium indologenes* (98.3 %), *Chryseobacterium jejuense* (98.1 %), *C. gleum* (98.1 %), *Chryseobacterium arthrosphaerae* (97.9 %), *C. joostei* (97.8 %), *C. hominis* (97.6 %), *Chryseobacterium oranimense* (97.5 %), *Chryseobacterium shigense* (97.5 %), *Chryseobacterium culicis* (97.4 %), *Chryseobacterium aquifrigidense* (97.2 %), *Chryseobacterium luteum* (97.0 %) and *C. scophthalmum* (97.0 %).

The 16S rRNA gene sequences of the type strains of all *Chryseobacterium* species with validly published names were retrieved from GenBank and aligned with the newly determined sequences by using the SeqTools program (Rasmussen, 2002). Phylogenetic trees were then constructed according to three different algorithms: neighbour-joining (Saitou & Nei, 1987), using the SeqTools and TreeView programs (Page, 1996; Rasmussen, 2002); maximum-likelihood, using the PHYML software package (Guindon & Gascuel, 2003); and maximum-parsimony, using version 4 of the MEGA software package (Kumar *et al.*, 2004). The evolutionary distances used for the neighbour-joining and maximum-likelihood trees were calculated by the Kimura two-parameter model (Kimura, 1980). Close-neighbour-interchange (search level=2, random additions=100) was applied in the maximum-parsimony analysis. The topology of each tree was evaluated by bootstrap analysis with 1000 replications (Felsenstein, 1985). In the neighbour-joining phylogenetic tree (Fig. 1), the novel strains clustered with members of the genus *Chryseobacterium*, forming a distinct subline within a subgroup of 17 *Chryseobacterium* species (including the type species *C. gleum*). Similar topologies were seen in the maximum-likelihood and maximum-parsimony trees (data not shown)

For the phenotypic characterization, minimum standards for the description of new taxa in the family *Flavobacteriaceae* were followed (Bernardet *et al.*, 2002). Several key characteristics, such as Gram staining, production of catalase and oxidase, and hydrolysis of Tween 80, L-tyrosine, aesculin, lecithin, starch, agar, gelatin and casein, were investigated using standard procedures (Smibert & Krieg, 1994). Hydrolysis of urea and DNA were tested as described by Bowman *et al.* (1996). Aerobic growth was assessed at 15, 25, 30, 37 and 42 °C in brain heart infusion (BHI) broth, and (at 25 °C) in BHI broth with 3.0, 4.5 and 6.5 % (w/v) added NaCl. Growth in BHI agar at 25 °C was also assessed under both anaerobic conditions (with 4–10 % CO_2) and micro-aerobic conditions (with 5–15 % O_2 and 5–12 % CO_2), which were created using the GasPak Plus and CampyPak Plus systems (BBL), respectively. Growth was also tested on MacConkey (bioMérieux), nutrient (Difco) and trypticase-soy (bioMérieux) agars. Gliding motility was investigated by the hanging-drop technique, and the production of flexirubin-type pigments and extracellular glycans were investigated as described by Bernardet *et al.* (2002). The novel strains were biochemically characterized further using the API 20NE and API ZYM systems (bioMérieux) according to the manufacturer's instructions, except that the incubation temperature was 25 °C. The type strains of 14 established species in the genus *Chryseobacterium* (*C. oncorhynchi* CECT 7794^T, *C. ureilyticum* CCUG 52546^T, *C. joostei* CCUG 46665^T, *C. jejuense* CCUG 61058^T, *C. gleum* CCUG 14555^T, *C. oranimense* CCUG 61056^T, *C. hominis* CCUG 52711^T, *C. indologenes* CCUG 14556^T, *C. arthrosphaerae* CCUG 57618^T, *C. shigense* CCUG 61059^T, *C. aquifrigidense* CCUG 61061^T, *C. luteum* CCUG 61057^T, *C. scophthalmum* CCUG 33454^T and *C. culicis* CCUG 58784^T) were used for reference in the investigations of the phenotypic, chemotaxonomic and genotypic properties of the novel strains and were grown under the same laboratory conditions as the novel strains. The five novel strains exhibited identical phenotypic characteristics except that strain 967B-08, unlike the other four novel strains,

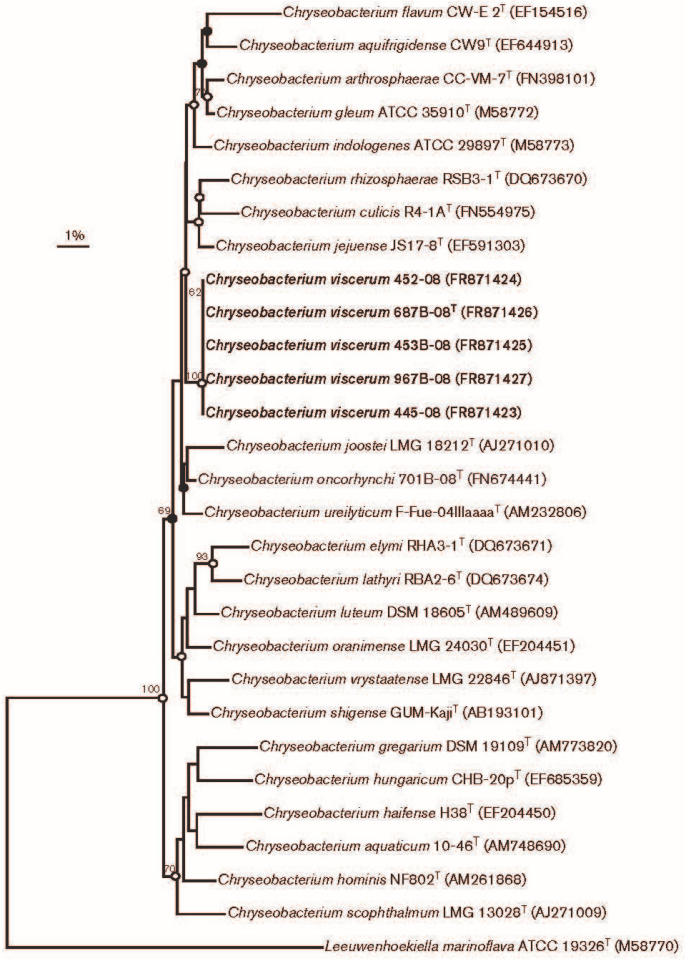

Fig. 1. A neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the relationship between the five novel strains from trout (strains 687B-08^T, 445-08, 452-08, 453B-08 and 967B-08) and closely related taxa. Percentage bootstrap values >70% (based on 1000 replications) are shown at branch points. Filled circles indicate that the corresponding nodes were also recovered in the maximum-parsimony tree. Open circles indicate that the corresponding nodes were also recovered in both the maximum-likelihood and maximum-parsimony trees. *Leeuwenhoekiella marinoflava* ATCC 19326^T (M58770) was used as an outgroup. Bar, 0.01 substitution per nucleotide position.

appeared to be devoid of esterase lipase (C8) activity. A detailed description of the morphological, physiological and biochemical characteristics of the novel strains is given in the species description and Table 1. Several phenotypic characteristics could be used to distinguish the novel strains not only from their nearest phylogenetic relative, *C. ureilyticum*, but also from other closely related species in the genus *Chryseobacterium* (Table 1).

Table 1. Phenotypic characteristics that differentiate strain 687B-08^T from the type strains of related members of the genus *Chryseobacterium*

Strains: 1, 687B-08^T; 2, *C. oncorhynchi* CECT 7794^T; 3, *C. ureilyticum* CCUG 52546^T; 4, *C. indologenes* CCUG 14556^T; 5, *C. jejuense* CCUG 61058^T; 6, *C. gleum* CCUG 14555^T; 7, *C. arthrosphaerae* CCUG 57618^T; 8, *C. joostei* CCUG 46665^T; 9, *C. hominis* CCUG 52711^T; 10, *C. shigense* CCUG 61059^T; 11, *C. oranimense* CCUG 61056^T; 12, *C. culicis* CCUG 58784^T; 13, *C. aquifrigidense* CCUG 61061^T; 14, *C. luteum* CCUG 61057^T; 15, *C. scophthalmum* CCUG 33454^T. All data are from this study, except the genomic DNA G+C contents of the reference strains. +, Positive; −, negative; W, weakly positive; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Growth at (°C):															
37	−	−	−	+	−	+	+	−	+	−	+	+	+	−	−
42	−	−	−	−	−	+	+	−	−	−	+[a*]	−	−	−	−
Tolerance to 3.0 % (w/v) NaCl	−	−	+[b]	+	+	+	+	+	−	+	+	+	−	−	+
Brown pigment on tyrosine agar	+	+	+	−	+	+	−	+	+	−	−	+	+	−	+
Precipitation on egg-yolk agar	+	−	+[b]	W	W	W	+	+	−	W	W	+	+[c]	+	−
Degradation of:									−						
Urea	+	+	+	+[d]	+	+[e]	+	+	−	−	−	+	−	−	−[f]
Tween 80	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+
Casein	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+
Starch	+	+	+[b]	+	+	+	+	+	−[g]	+	−[a]	+	−[c]	−[h]	−
Gelatin	+	+	+	+	+	+	+	+	−[g]	+	+	+	−[c]	+	−[f]
Tyrosine	+	+	+	−	+	−[e]	+	+	−	−	+	−[i]	−	−	+
Nitrate reduction	−	−	−	−[d]	−	+[e]	+	−	+	−	−	−	+	−	−
Indole production	+	+	+	+	+[j]	+[e]	+	+	+	+	+	+	−	+	−
Enzyme activity:															
β-Galactosidase	−	−	−	+	−	+	−	−	−	−	+	−	−	+	−
Esterase (C4)	−	−	−[b]	+	+	−	+	−	−	−	+	+	−	−	−[f]
Esterase lipase (C8)	+	+	−	+	+	+	−	+	+	+	+	−	−[c]	−[h]	+
Valine arylamidase	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+
Trypsin	+	+	−[b]	−	+	−	+	+	−[g]	+	−	−	−	+	+
α-Glucosidase	+	+	−	−	−[j]	−	+	−[k]	−	−	−	−	−	−[h]	+
β-Glucosidase	−	−	−	−	−	+	−	−	−	+	+	−	−	+	−[f]
N-Acetyl-β-glucosaminidase	+	−	−[b]	+	−[j]	−	+	+	−	−	+[a]	−	−[c]	−[h]	+
Assimilation of:															
Arabinose	−	−	−	−	−	+	−	−	−	−	+	+[i]	+[c]	+[h]	−
Mannose	+	+	+[b]	+	+	+	+[l]	+	+	+	+	+	−	+[h]	+[f]
Mannitol	+	−	−	−	−	−	+[l]	−	−	+	−	+[i]	−	−	−
Maltose	+	+	+	+	+	+	+[l]	+	+	+	+	+	−[c]	+[h]	−[f]
Adipic acid	−	−	−	−	−	+	−	−	−	−	+	−	+	−	−
Citrate	+	+	−	+	+[j]	+[e]	−	−	−	−	+	+[i]	+[c]	−	−
DNA G+C content (mol%)†	38.6	36.3	39.2	38.5	37.0	38.0	ND	36.8	36.5	36.6	36.1	ND	35.6	ND	34.2

*Opposite results were reported by: *a*, Hantsis-Zacharov *et al.* (2008); *b*, Herzog *et al.* (2008); *c*, Park *et al.* (2008); *d*, Yabuuchi *et al.* (1983); *e*, Holmes *et al.* (1984); *f*, Mudarris *et al.* (1994); *g*, Vaneechoutte *et al.* (2007); *h*, Behrendt *et al.* (2007); *i*, Kämpfer *et al.* (2010b); *j*, Weon *et al.* (2008); *k*, Hugo *et al.* (2003); *l*, Kämpfer *et al.* (2010a).

†Data for DNA G+C content for strains 1–15 are as follows: 1, this study; 2, Zamora *et al.* (2012); 3, Herzog *et al.* (2008); 4, 6, 8, 15, Bernardet *et al.* (2011); 5, Weon *et al.* (2008); 7, Kämpfer *et al.* (2010a); 9, Vaneechoutte *et al.* (2007); 10, Shimomura *et al.* (2010); 11, Hantsis-Zacharov *et al.* (2008); 12, Kämpfer *et al.* (2010b); 13, Park *et al.* (2008); 14, Behrendt *et al.* (2007).

DNA–DNA hybridizations were carried out both between the five novel strains and between strain 687B-08^T, chosen as a representative isolate, and the type strains of closely related established species in the genus *Chryseobacterium* (i.e. those showing 16S rRNA gene sequence similarities with strain 687B-08^T of >97.0 %). For this, DNA was extracted and purified by the method of Marmur (1961) and hybridizations were carried out, under optimal conditions and a temperature of 45 °C (De Ley & Tijtgat, 1970), by the membrane method (Johnson, 1994; Arahal *et al.*, 2001). DNA–DNA relatedness values were calculated as described by Johnson (1994). The results reported are the mean values from the three independent experiments that were carried out for each analysis. Since the DNA–DNA relatedness values recorded between them were very high (99–100 %), the five novel strains are considered to be

members of a single species (Wayne *et al.*, 1987). However, the relatively low DNA–DNA relatedness values recorded between strain 687B-08^T and *C. oncorhynchi* CECT 7794^T (57 %), *C. ureilyticum* CCUG 52546^T (47 %), *C. joostei* CCUG 46665^T (35 %), *C. jejuense* CCUG 61058^T (51 %), *C. gleum* CCUG 14555^T (52 %), *C. hominis* CCUG 52711^T (33 %), *C. oranimense* CCUG 61056^T (45 %), *C. indologenes* CCUG 14556^T (40 %), *C. arthrosphaerae* CCUG 57618^T (31 %), *C. shigense* CCUG 61059^T (45 %), *C. aquifrigidense* CCUG 61061^T (42 %), *C. luteum* CCUG 61057^T (22 %), *C. culicis* CCUG 58784^T (50 %) and *C. scophthalmum* CCUG 33454^T (21 %) indicate that strain 687B-08^T belongs to a novel species (Wayne *et al.*, 1987; Rosselló-Mora, 2006).

By using the mid-point values of thermal denaturation profiles (Marmur & Doty, 1962) determined in a UV-Vis spectrophotometer (Perkin-Elmer) at 260 nm, the genomic DNA G+C contents of strains 687B-08^T, 445-08, 452-08, 453B-08 and 967B-08 were found to be 38.6, 38.2, 38.8, 39.0 and 38.6 mol%, respectively. These values fall within the range reported for established species in the genus *Chryseobacterium* (Bernardet *et al.*, 2006, 2011).

The respiratory quinones of strain 687B-08^T were identified by the Identification Service of the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ), at Braunschweig, Germany, and by Dr Brian Tindall (also of the DSMZ). The quinones were separated by TLC on silica-gel plates and then further analysed by HPLC. The major quinone of strain 687B-08^T was identified as MK-6 (the major quinone of all members of the family *Flavobacteriaceae*; Bernardet & Nakagawa, 2006). MK-5 was also detected in cells of strain 687B-08^T but only as a minor component.

The cellular fatty acids of strain 687B-08^T and 13 type strains of the most closely related *Chryseobacterium* species were investigated by using an HP 5890 GC (Hewlett Packard), cells that had been grown for 40±6 h on blood agar at 30 °C under aerobic conditions, and a standardized protocol (http://www.ccug.se/pages/CFA_method_2008.pdf) similar to that of the Sherlock Microbial Identification System (MIDI). The fatty acid profile of strain 687B-08^T, in which iso-$C_{15:0}$ (40.9 %), iso-$C_{17:1}\omega9c$ (18.5 %), $C_{16:1}\omega6c$ (12.3 %) and iso-$C_{17:0}$ 3-OH (14.7 %) predominated (Table 2), was similar to those of closely related (Table 2) and less closely related (Vandamme *et al.*, 1994; Bernardet *et al.*, 2005, 2011) members of the genus *Chryseobacterium*.

Based on the phenotypic, chemotaxonomic and phylogenetic evidence and the results of the DNA–DNA hybridizations, the five novel strains from rainbow trout represent a single novel species in the genus *Chryseobacterium*, for which the name *Chryseobacterium viscerum* sp. nov. is proposed.

Description of *Chryseobacterium viscerum* sp. nov.

Chryseobacterium viscerum (vis.ce'rum. L. gen. pl. n. *viscerum* of the inner parts of the body, internal organs, viscera).

Table 2. Cellular fatty acid contents (%) of strain 687B-08^T and the type strains of related species in the genus *Chryseobacterium*

Strains: 1, 687B-08^T; 2, *C. oncorhynchi* CECT 7794^T; 3, *C. ureilyticum* CCUG 52546^T; 4, *C. indologenes* CCUG 14556^T; 5, *C. jejuense* CCUG 61058^T; 6, *C. gleum* CCUG 14555^T; 7, *C. arthrosphaerae* CCUG 57618^T; 8, *C. joostei* CCUG 46665^T; 9, *C. hominis* CCUG 52711^T; 10, *C. shigense* CCUG 61059^T; 11, *C. oranimense* CCUG 61056^T; 12, *C. culicis* CCUG 58784^T; 13, *C. aquifrigidense* CCUG 61061^T; 14, *C. luteum* CCUG 61057^T; 15, *C. scophthalmum* CCUG 33454^T. All data are from this study, generated from samples of biomass that were produced under the same conditions. Fatty acids amounting to less than 1 % in all strains are not shown. TR, Trace amount (<1 %); –, not detected.

Fatty acid	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
iso-$C_{13:0}$	TR	TR	1.8	–	1.4	–	TR	1.3	7.3	1.4	–	1.3	–	1.6	TR
iso-$C_{15:0}$	40.9	33.7	42.5	37.2	38.6	37.2	37.6	41.4	43.4	41.7	42.0	46.2	37.1	44.7	41.8
iso-$C_{15:0}$ 3-OH	2.9	3.5	3	3.0	2.9	2.3	2.4	2.9	4.4	3.1	3.1	2.8	3.1	2.5	3.2
anteiso-$C_{15:0}$	TR	TR	–	–	–	–	–	–	7.3	TR	–	–	–	5.7	1.0
$C_{16:0}$	1.8	1.4	TR	2.1	1.6	1.8	1.9	1.3	–	1.2	1.4	2.4	2.8	–	1.2
$C_{16:0}$ 3-OH	TR	TR	TR	–	TR	–	TR	TR	–	TR	–	TR	2.2	–	–
$C_{16:1}\omega6c$	12.3	16.0	11.7	12.1	16.0	14.8	12.4	12.0	7.2	19.6	14.0	11.3	19.3	8.5	11.7
iso-$C_{17:0}$	1.1	1.0	TR	–	–	1.6	1.5	1.2	–	–	–	2.0	–	–	TR
iso-$C_{17:1}\omega9c$	18.5	24.6	23.3	26.8	18.4	23.3	24.1	20.6	12.8	16.0	21.5	11.5	18.2	21.4	20.4
iso-$C_{17:0}$ 3-OH	14.7	14.1	11.0	13.1	14.5	13.1	14.1	13.2	11.6	12.0	16.0	17.1	13.2	14.1	11.5
$C_{18:1}\omega5c$	–	–	–	TR	–	–	–	–	1.2	–	–	–	–	–	–
Summed feature 2*	2.6	1.9	–	2.4	2.4	2.7	1.6	1.6	TR	2.1	1.9	2.0	2.4	–	1.7
Unknown fatty acids†															
ECL 13.566	TR	TR	3.3	3.3	1.1	1.6	2.2	2.7	2.2	1.3	–	1.1	1.0	1.7	5.6
ECL 16.580	1.1	1.1	1.0	–	1.1	1.0	TR	1.0	–	–	–	1.4	–	–	–

*Summed features represent groups of two or three fatty acids that could not be resolved by GLC with the protocol that was used. Summed feature 2 contained $C_{18:2}\omega6,9c$ and/or anteiso-$C_{18:0}$.
†ECL, Equivalent chain-length.

Cells are Gram-staining-negative rods that measure approximately 0.8 μm in diameter and 2.4 μm in length and are non-spore-forming and non-gliding. Grows well under aerobic conditions and weakly under micro-aerobic conditions; does not grow under anaerobic conditions. Grows in BHI broth at 15–30 °C (optimum 25 °C) but not at 37 °C. After incubation at 25 °C for 72 h, growth is detectable on TGE, trypticase-soy and nutrient agars but not on MacConkey agar. Growth does not occur in BHI broth containing 3.0, 4.5 or 6.5 % (w/v) added NaCl. After incubation at 25 °C on TGE agar for 72 h, colonies are circular, pale orange, smooth and entire. Colonies are haemolytic on Columbia agar after 72 h of incubation at 25 °C. Flexirubin-type pigments are produced. Positive for catalase and oxidase activities and the hydrolysis of starch, L-tyrosine, gelatin, casein, aesculin, urea, Tween 80 and lecithin but negative for the reduction of nitrate and nitrite and the hydrolysis of agar, arginine and DNA. A brown pigment is produced on tyrosine agar. Indole is produced but not H_2S. Acid is not produced from D-glucose. Utilizes glucose, mannose, mannitol, maltose and citrate as sole carbon and energy sources but not arabinose, *N*-acetyl-D-glucosamine, gluconate, caprate, adipate or malate. Positive for alkaline phosphatase, leucine arylamidase, valine arylamidase, trypsin, acid phosphatase, α-glucosidase, naphthol-AS-BI-phosphohydrolase and *N*-acetyl-β-glucosaminidase activities but negative for esterase (C4), lipase (C14), cystine arylamidase, α-chymotrypsin, α-galactosidase, β-glucuronidase, β-glucosidase, α-mannosidase, β-galactosidase and α-fucosidase activities. Esterase lipase (C8) activity is strain-dependent. Cells contain MK-6 as the major respiratory quinone, with minor amounts of MK-5 also present. The major cellular fatty acids are iso-$C_{15:0}$, iso-$C_{17:1}\omega9c$, $C_{16:1}\omega6c$ and iso-$C_{17:0}$ 3-OH.

The type strain, 687B-08^T (=CECT 7793^T =CCUG 60103^T), was isolated from the gills of a diseased rainbow trout. The genomic DNA G+C content of the type strain is 38.6 mol%.

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RESEARCH ARTICLE Open Access

First isolation and characterization of *Chryseobacterium shigense* from rainbow trout

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Abstract

Background: There have been an increasing number of infections in fish associated with different species of *Chryseobacterium*, being considered potentially emerging pathogens. Nevertheless the knowledge of the diversity of species associated with fish disease is partial due to the problems for a correct identification at the species level based exclusively on phenotypic laboratory methods.

Results: *Chryseobacterium shigense* was isolated from the liver, kidney and gills of diseased rainbow trout in different disease episodes that occurred in a fish farm between May 2008 and June 2009. Identity of the isolates was confirmed by 16 S rRNA gene sequencing and phenotypic characterization. Isolates represented a single strain as determined by random amplified polymorphic DNA analysis.

Conclusions: This is the first description of the recovery of *C. shigense* from clinical specimens in trout, a very different habitat to fresh lactic acid beverage where it was initially isolated.

Background

Members the genus *Chryseobacterium* are widely distributed microorganisms that can be recovered from a wide variety of environments, such as fresh water, sewage and wastewater, soil or food sources, such as milk, poultry and meat and dairy products [1]. Some species of *Chryseobacterium* have been involved in human infections, acting as sporadic but severe opportunistic nosocomial pathogens [2,3]. In veterinary medicine, chryseobacteria are not relevant pathogens for domestic animals, but they are widely distributed in aquatic environments and fish farms [1,4]. Until recently members of the genus *Chryseobacterium* were not commonly associated with fish infections. However, there has been an increase in the frequency of clinical cases in which *Chryseobacterium* sp. strains have been isolated from different fish species. Thus, *Chryseobacterium balustinum*, *Chryseobacterium scophtalmum* and *Chryseobacterium joostei* have been isolated from diseased fish [4-6]. More recently, *Chryseobacterium piscicola* has been reported to produce mortalities in farmed Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) in Chile and Finland [7-9], *Chryseobacterium arothri* was isolated from the kidneys of the pufferfish *Arothron hispidus* in Hawaii [10] and *Chryseobacterium chaponense* from diseased farmed Atlantic salmon in Chile [11]. In fact, some *Chryseobacterium* species are considered potentially emerging pathogens in fish [4]. However, many chryseobacteria isolated from diseased fish are usually identified only at the genus level due to the difficulty of their correct identification by phenotypically based laboratory methods alone [4,5], which limits the knowledge of the diversity of species associated with fish disease.

Methods

Bacterial strains and culture conditions

The bacterial isolates were recovered from liver (635–08, 628-2-08; 692–08), kidney (664–09) and gills (706B-08, 972B-08, 1107B-09) of rainbow trout (*Oncorhynchus mykiss*) fry during five outbreaks (May, June and September of 2008 and June 2009) occurred in a fish farm located in the central region of Spain. The fish farm had a flow-through system with intake of water from an adjacent river. The water temperature is quite constant during the whole year (15 °C ± 1). Stocking densities

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vary along the growth period from 3 to 20 kg/m^3, with a water exchange rate of 15–20 min. Feed consist in a commercial brand of extruded micropelets. Average feeding rate is about 3 % of biomass, delivered in four times by hand. Based on clinical symptoms and epidemiological background (*Flavobacterium psychrophilum* had previously been isolated from the farm) rainbow trout fry syndrome (RTFS) was suspected. Trout were submitted alive to the Animal Health Surveillance Centre (VISAVET) of the Universidad Complutense of Madrid for a confirmatory microbiological diagnosis. Trout were euthanized and necropsied under aseptic conditions. Samples of liver, kidney and gills were incubated on Anacker and Ordal´s agar for 7 days at 14 °C. Nutrient agar was used for routinely growth of clinical isolates after their initial isolation. Stock cultures were preserved at −80 °C in a cryopreservative media composed of tryptone (2.5 %), unskimed milk (5 %) and glicerine (20 %).

F. psychrophilum PCR assay

The PCR assay specific for *F. psychrophilum* was performed as described by Wiklund et al. [12].

16 S rRNA gene sequencing

The 16 S rRNA gene of the seven isolates was amplified and sequenced as described previously [13] and subjected to a comparative analysis. A nearly complete 16 S rRNA gene fragment (>1,400 bp) was obtained bidirectionally using the universal primers pA (5'-AGAGTTT GATCCTGGCTCAG; positions 8–27, *Escherichia coli* numbering) and pH* (5'-AAGGAGGTGATCCAGCC GCA; positions 1,541-1,522, *E. coli* numbering). The determined sequences were compared with the sequences of other Gram-negative species available in the GenBank database, by using the FASTA program (http://www.ebi.ac.uk/fasta33). Phylogenetic relationships were inferred using the neighbor-joining algorithm as described previously [14].

Random amplified polymorphic DNA fingerprinting

For all strains genomic DNA was prepared using method described by Marmur [15]. The primers used for RAPD-PCR were P1 (5'-CTGCTGGGAC-3') and P2 (5'-CGC CCTGCCC-3') (Roche Diagnostics S.L.) described previously (3). PCR amplifications were performed using a commercial PCR master mix (kit QIAGEN Multiplex PCR) adding the DNA template (5 μl), 0.5 μM of each primer and water up to a final volume of 25 μl. PCR amplifications were carried out in a Mastercycler gradient thermocycler (Eppendorf) with the following parameters: an initial denaturalization of 15 min at 95 °C and 30 cycles of 1 min at 94 °C, 1 min at 36 °C, and 2 min at 72 °C. PCR-amplified products (20 μl) were separated at 60 V for 2 h in 1.5 % agarose gel electrophoresis supplemented with 1X Syber safe® (Invitrogen, Eugene, OR). DNA banding patterns were analyzed using bio-Numerics software (Applied Maths) to calculate Dice coefficients of correlation and to generate a dendrogram using the unweighted pair group method of arithmetic averages (UPGMA) clustering. To assess the repeatability of RAPD-PCR, isolates were submitted to three different amplifications assays for each primer, realized in different days and in similar conditions as described above.

Phenotypic analysis

Isolates were characterized using conventional phenotypic tests proposed by Bernardet et al. [16] i.e. production of catalase and oxidase, motility, hydrolysis of agar, casein, L-tyrosine, aesculin, DNA, urea, gelatin and starch; production of flexirubin-type pigments; growth on MacConkey (bioMérieux) and nutrient (Difco) agars and growth at 15, 25, 30, 37 and 42 °C, with 3.0, 4.5 and 6.5 % added NaCl, and under anaerobic and microaerobic conditions, were determined as described previously [17]. The isolates were further biochemically characterized using the API 20NE and API ZYM systems (bioMérieux) according to the manufacturer's instructions, except that incubation temperature was 25 °C. The type strain of *C. shigense* CCUG 61059^T = DSM 17126^T was used as a reference in all tests.

Results

All isolates gave shiny, round, yellow-pigmented colonies on Anacker and Ordal agar, a characteristic that lead to the presumptive diagnosis of infection by *F. psychrophilum*, but none of the isolates gave the expected amplicon product of 1,089 bp, specific of *F. psychrophilum*. Moreover, cells of trout isolates were straight short Gram negative rods after Gram staining. Comparative analysis of the 16 S rRNA gene sequences revealed that the isolates shared 99.8-100 % sequence similarity between each other, thus demonstrating their high phylogenetic relatedness, 99.2-99.8 %, with the type strain of *C. shigense* (GUM-Kaji^T; Figure 1) and only 81.8-81.9 % with *F. psychrophilum* NCIMB 1947^T (GenBank accession n D12670). The 16 S rRNA gene sequences of the isolates included in this study have been deposited in GenBank under the accession numbers indicated in Figure 1.

Phenotypically all trout isolates were catalase and oxidase positive, non-motile, grew on nutrient agar with yellow and shiny colonies but not on MacConkey agar, produced flexirubin-type pigment, were able to grow at 5–30 °C but not at 37 °C, and hydrolysed starch, casein and gelatin. With the API 20NE system they exhibited homogeneous biochemical characteristics displaying the numerical profiles 3452205, while *C. shigense* CCUG 61059^T gave the numerical profile 2456204. With the APY ZYM strips, the trout isolates, as well as the type

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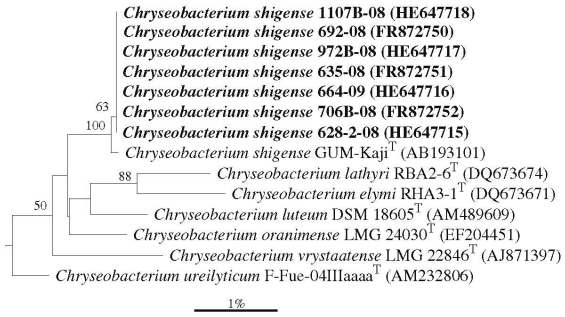

Figure 1 Phylogenetic relationships of the clinically trout isolates and close related species in the genus *Chryseobacterium* inferred using the neighbor-joining method with 16 S rRNA gene sequences. Bootstrap values (expressed as a percentage of 1000 replications) >50 % are given at the branching points. *Leeuwenhoekiella marinoflava* ATCC 19326^T (M58770) was used as outgroup. Bar, 1 % sequence divergence.

strain of *C. shigense* CCUG 61059^T, expressed activity for alkaline phosphatase, leucine arylamidase, trypsin, acid phosphatase and naphthol-AS-BI-phophohydrolase but not for esterase C4, lipase C14, cystine arylamidase, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. Clinical isolates of *C. shigense* expressed activity for valine arilamidase and not for ester lipase C8 and β-glucusidase, while *C. shigense* CCUG 61059^T gave opposite results for these tests.

After genetic characterization by random amplified polymorphic DNA, both oligonucleotides generated reproducible patterns, but an appropriate number on bands were produced with oligonucleotide P2 (Figure 2). The seven *C. shigense* trout isolates showed undistinguishable RAPD fingerprints with amplifications bands ranging from 600 to 2500 bp, indicating genetic homogeneity among them. On the other hand, the strain CCUG 61059^T yielded a completely different fingerprint (Figure 2).

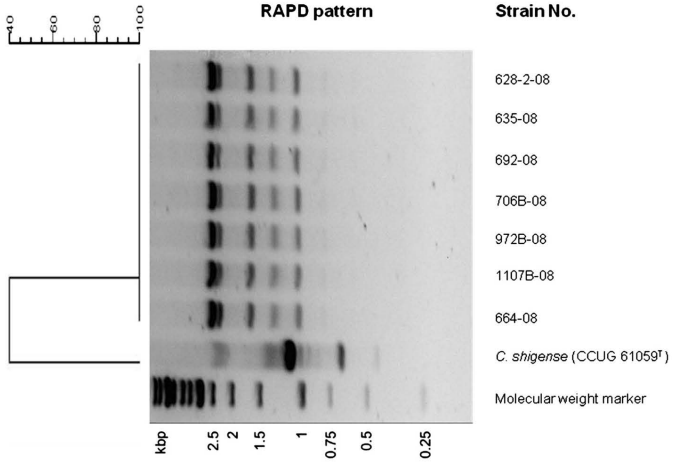

Figure 2 RAPD profiles obtained with primer P2 for the *C. shigense* strains and the dendrogram generated based on the Dice coefficient using the BioNumerics software (Applied Maths).

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Discussion

Diagnosis of bacterial fish diseases is not possible purely on the basis of the clinical signs and symptoms observed in diseased fish, because many of them can be caused by more than one etiological agent. Presumptive diagnosis is usually based on previous epidemiological data and a rough bacteriological analysis of cultured organisms from affected animals. In the present study, isolates were recovered from diseased trout suspected of *F. psychrophilum* infection because the fish farm had previous records of infections with this fish pathogen and the macroscopic characteristics of the colonies obtained on Anacker and Ordal agar were also compatible with that presumptive diagnosis. However, cell morphology of the trout isolates following Gram staining was different of the filamentous rods exhibited by *F. psychrophilum* [18], they were non-motile and none gave a positive reaction with a *F. psychrophilum* species-specific PCR assay [13]. This lack of amplification is consistent with the low similarity observed (81.8-81.9 %) between the 16 S rRNA gene sequences of trout isolates and *F. psychrophilum* NCIMB 1947^T. The trout isolates exhibited the highest 16 S rRNA sequences similarities with *C. shigense* GUM-Kaji^T (99.2-99.8 %), percentages higher than the 99 % sequence similarity used as the criterion for species identification [19]. In addition, most of the phenotypic characteristics of the *C. shigense* trout isolates were consistent with the current description of this species based solely in the strain DSM 17126^T [20], which support the identification based on 16 S rRNA gene sequencing. Despite the overall phenotypic similitude, some differences were found between the *C. shigense* trout isolates and the type strain *C. shigense* CCUG 61059^T. Thus, trout isolates reduced nitrate, assimilated citrate but not mannitol and produce the enzyme valine arylamidase but not the enzymes ester lipase C8 and β-glucosidase, while the type strain of *C. shigense* CCUG 61059^T gave opposite reactions for these traits.

Widely spread microorganisms are usually genetically heterogeneous [21]. Members of the genus *Chryseobacterium* are isolated from a wide range of environments [1] and therefore, it could be expected to be genetically diverse. However, trout isolates exhibited undistinguishable RAPD fingerprints indicating that they represent a single strain. This fact, together with their isolation in pure culture from internal organs might suggest a clinical significance; however the role of *C. shigense* as the causative agent of the disease episodes in trout cannot be undoubtedly established in absence of experimental infections trials.

Some members of the family *Flavobacteriaceae*, as *Chryseobacterium*, usually are opportunistic pathogens [4], because they require the existence of different predisposing factors such as coinfections with other bacteria or virus [7] or other environmental or husbandry sources of stress. No infestations or viral infections were detected previously to the disease episodes, but these usually occurred after transportation of rainbow trout fry from the hatchery to fish farm, or in tanks with elevated stock densities. These circumstances represent stressful conditions for fish [22] and might have increased the susceptibility of fry trout to infection.

Several species of *Chryseobacterium*, such as *C. piscicola* [8], *C. chaponense* [11], and *C. shigense* in this study, have been isolated from diseased fish in which *F. psychrophilum* infections were initially suspected. Although there are no clear evidences for considering these species as consistent pathogens for fish, they should be considered for a differential diagnosis in those cases with a suspicious of *F. psychrophilum* infection. Table 1 shows some phenotypic characteristics that can be useful for their differentiation.

Since the initial description of *C. shigense* from a fresh lactic acid beverage in Japan [20], no further isolations of this species have been reported. Consequently, the isolation of *C. shigense* from trout shows that it can also occur in a very different habitat. To our knowledge, this is the first description of the isolation of *C. shigense* from clinical specimens.

Conclusions

In this work we describe by first time the recovery of *C. shigense* from clinical specimens in trout, showing

Table 1 Phenotypic characteristics[a] that can be useful to differentiate the species *C. shigense*, *C. piscicola*, *C. chaponense* and *F. psychrophilum*

Characteristic	*C. shigense*	*C. piscicola*	*C. chaponense*	*F. psychrophilum*[b]
Growth at/with:				
37 C	-	-	+	-
3.0 % NaCl	w	+	-	-
Flexirubin-type pigment	+	+	-	+
Hydrolysis of:				
Casein	+	-	-	+
L-tyrosine	+	-	-	V
Gelatin	+	+	-	+
Starch	+	+	-	-
DNA	-	w	-	-
Enzyme activity:				
α-glucosidase	-	+	+	V
β -glucosidase	-	+	+	V
β-galactosidase	-	-	-	V

[a] Data for *C. shigense* are taken from this study, for *C. piscicola* from reference Ilardi et al. [8], for *C. chaponense* from reference Kämpfer et al. [11] and for *F. psychrophilum* from references Bernardet et al. [23], Cipriano and Holt [18] and Hesami et al. [24]. [b] *F. psychrophilum* differs also from *C. shigense* by its inability to utilize glucose as sole carbon source, to hydrolyze aesculin and to produce a brown diffusible pigment on tyrosine agar. +, Positive reaction; -, negative reaction; V, variable reaction; w, weak reaction.

that it can also occur in a very different habitat to fresh lactic acid beverage where it was initially isolated.

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Authors' contributions
LZ carried out the phenotypic and genetic analyses and participated in the analysis of the data and drafting of the manuscript. AIV participated in the design of the study, in phylogenetic analysis and drafting of the manuscript. MAP participated in the recovery of clinical specimens. LD participated in the design of the study. JFJG conceived the study and participated in its design, drafting and coordination. All the authors read and approved the final manuscript.

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Discusión

6 Discusión

Los géneros *Flavobacterium* y *Chryseobacterium* pertenecen a la familia *Flavobacteriaceae* y están formados por microorganismos muy ubicuos que se aíslan, entre otras muchas fuentes, de ambientes acuáticos (Bernardet y Bowman, 2006; Bernardet y col., 2006). Aunque la mayoría de las especies incluidas en ambos géneros son saprofitas, algunas especies como *F. branchiophilum*, *F. columnare* y *F. psychrophilum* son reconocidas como importantes patógenos de peces (Ostland y col., 1994; Barnes y Brown 2011; Declercq y col., 2013) y otras, como *C. scophthalmum*, *C. piscicola*, *C. piscium*, *C. chaponense*, *F. johnsoniae*, *F. hydatis*, *F. succinicans*, *F. chilense, F. araucananum* y *F. spartansii,* se han aislado de peces enfermos (Mudarris y col., 1994; de Beer y col., 2006; Flemming y col., 2007; Ilardi y col., 2009; Kamfer y col., 2012; Loch y Faisal 2014). En el presente trabajo de Tesis Doctoral hemos realizado un estudio taxonómico palifásico de un grupo de bacterias Gram negativas aisladas de huevos embrionados y de alevines enfermos de trucha arcoíris que presentaban síntomas clínicos compatibles con el síndrome del alevín de la trucha arcoíris, una enfermedad septicémica cuyo agente etiológico es *Flavobacterium psychrophilum* y que afecta a alevines juveniles en los que provoca un cuadro septicémico agudo con una intensa inflamación del bazo (esplenomegalia) y elevada mortalidad (Austin y Stobie, 1991).

Los aislados estudiados (Tabla 6) se obtuvieron en agar *Anacker and Ordal,* uno de los medios de cultivo empleados comúnmente para el aislamiento de *F. psychrophilum* (Anacker y Ordal, 1959). Este hecho, junto con los datos clínicos hizo sospechar inicialmente que pudiera tratarse de esta especie bacteriana. Sin embargo, tras la tinción de Gram, ninguno de estos microorganismos presentó la típica morfología celular de bacilos filamentosos que exhibe *F. psychrophilum* (Cipriano y Holt, 2005) y tampoco fue posible su identificación mediante una PCR específica para este patógeno (Wiklund y col., 2000). Debido a que estos microorganismos fueron aislados mayoritariamente de alevines enfermos y en algunos casos se obtuvieron en cultivo puro, consideramos que podrían tener una significación clínica en el proceso infeccioso que padecían los alevines y por ello decidimos realizar un estudio exhaustivo de los mismos con la finalidad de poder identificarlos correctamente.

Tabla 6. Relación de aislados bacterianos estudiados, especificando fecha, órgano de aislamiento y color de la colonia

Fecha de aislamiento	Referencia	Órgano de aislamiento	Color de colonia
08/05/2008	424-08	Hígado	Amarilla
	425B-08	Branquias	Amarilla
	426B-08	Branquias	Amarilla
	433B-08	Branquias	Amarilla
	435-08^{T}	Hígado	Amarilla
	437B-08	Branquias	Amarilla
	441B-08	Branquias	Amarilla
	445-08	Hígado	Naranja
	449-08	Hígado	Naranja
	449B-08	Branquias	Naranja
	452-08	Hígado	Naranja
	453B-08	Branquias	Naranja
30/05/2008	628-1-08	Hígado	Amarilla
	628-2-08	Hígado	Naranja
	631-08^{T}	Hígado	Amarilla
	635-08	Hígado	Naranja
	646-08	Hígado	Amarilla
	646B-08	Branquias	Amarilla
	650-08	Hígado	Amarilla
04/06/2008	687B-08^{T}	Branquias	Naranja
	688B-08	Branquias	Amarilla
	692-08	Hígado	Naranja
	695B-08	Branquias	Amarilla
	698-2-08	Hígado	Naranja
	701B-08^{T}	Branquias	Naranja
	706B-08	Branquias	Naranja
	711B-08	Branquias	Naranja

Tabla 6. Continuación

Fecha de aislamiento	Referencia	Órgano de aislamiento	Color de colonia
16/09/2008	947B-08	Branquias	Amarilla
	950B-08	Branquias	Amarilla
	967B-08	Branquias	Naranja
	972B-08	Branquias	Naranja
	978B-08	Branquias	Amarilla
	983-08^{T}	Hígado	Amarilla
	986-08	Hígado	Amarilla
	991-08	Hígado	Amarilla
23/10/2008	1083-08	Hígado	Naranja
	1084-08^{T}	Hígado	Naranja
	1084B-08	Branquias	Amarilla
	1095B-08	Branquias	Naranja
	1107B-08	Branquias	Naranja
24/10/2008	1126-1H-08^{T}	Huevos embrionados	Amarilla
16/01/2009	22B-09	Branquias	Amarilla
29/01/2009	47B-2-09	Branquias	Amarilla
	47B-3-09	Branquias	Amarilla
	51B-09	Branquias	Amarilla
	53B-3-09	Branquias	Amarilla
	55B-09	Branquias	Amarilla
	56B-1-09	Branquias	Amarilla
	57B-2-09^{T}	Branquias	Amarilla
	58B-1-09	Branquias	Amarilla
	59B-3-09^{T}	Branquias	Amarilla
	60B-3-09	Branquias	Amarilla
	60B-4-09	Branquias	Amarilla
	61B-09	Branquias	Amarilla
	62B-2-09	Branquias	Amarilla

Tabla 6. Continuación

Fecha de aislamiento	Referencia	Órgano de aislamiento	Color de colonia
21/04/2009	412R-09^T	Riñón	Amarilla
03/06/2009	662-09	Hígado	Amarilla
	664R-09	Riñón	Naranja
	666-09	Hígado	Amarilla
22/07/2009	976H-09	Huevos embrionados	Amarilla
	977H-09	Huevos embrionados	Amarilla

Teniendo en cuenta el color de las colonias, los aislados se dividían en dos grupos. Así, un grupo crecía dando lugar a colonias amarillas, mientras que el otro formaba colonias de color naranja (Tabla 6). La primera aproximación a la identificación de estos microorganismos la realizamos mediante la identificación molecular basada en la secuenciación del gen que codifica para el 16S ARNr puesto que la identificación bioquímica de las flavobacterias es difícil debido a la complejidad y diversidad de estos microorganismos (Pickett 1989; Tiirola y col., 2002). La secuenciación de este gen es muy útil para la correcta identificación de bacterias atípicas o inusuales a partir de muestras clínicas (Bosshard y col., 2006; Janda y Abbott, 2007) y además se considera también una técnica recomendable para la identificación de este grupo de microorganismos (Elkamel y Mohamed, 2012; Mun y col., 2013).

El análisis de las secuencias del 16S ARNr de los aislados permitió la adscripción de los mismos a los géneros *Flavobacterium* y *Chryseobacterium*. Los aislados que formaron colonias amarillas constituyeron siete grupos filogenéticos (F1-F7) que mostraron porcentajes de similitud en la secuencia de dicho gen mayores del 98,9% con diferentes especies del género *Flavobacterium* (Fig. 5), mientras que los aislados que formaron colonias naranja se agruparon formando cuatro grupos filogenéticos (C1-C4) que presentaron porcentajes de similitud en la secuencia del gen 16S ARNr mayores del 99,1% con distintas especies del género *Chryseobacterium* (Fig. 6).

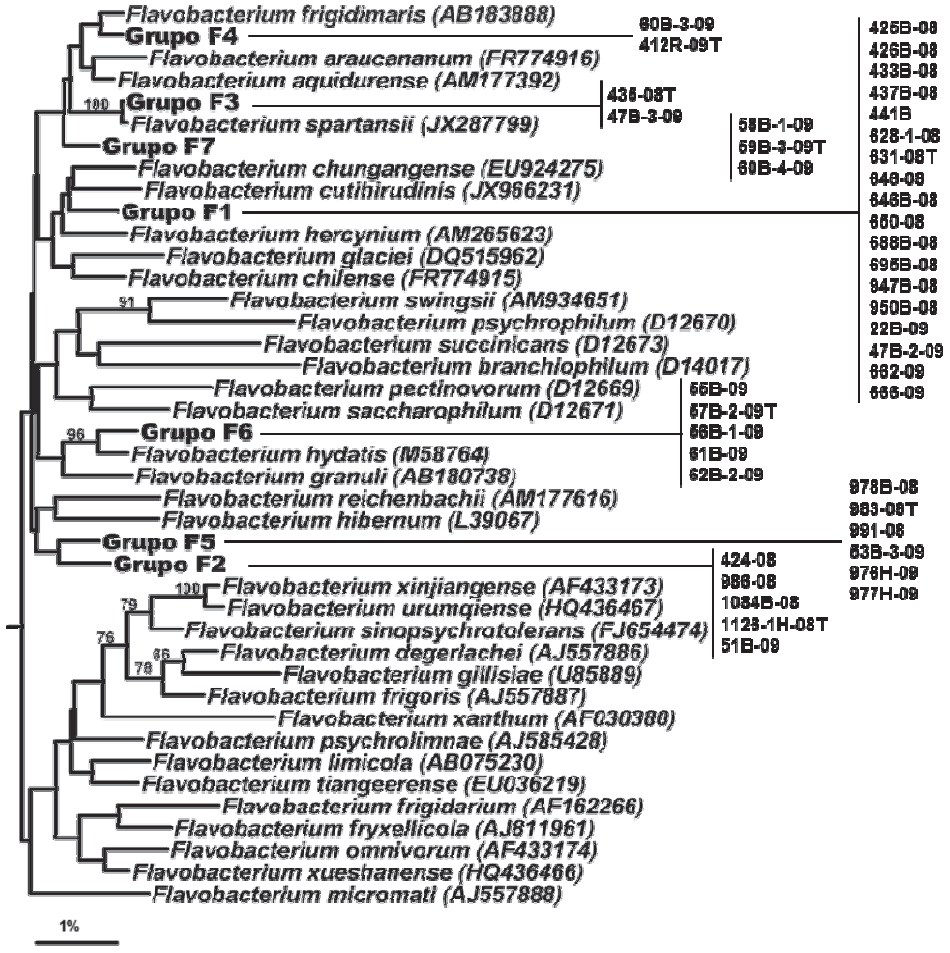

Figura 5. Árbol filogenético basado en la comparación de secuencias del gen 16S ARNr mediante el algoritmo *Neighbour Joining* en el que se muestra la posición de los aislados de trucha en relación con las especies filogenéticas más próximas del género *Flavobacterium*. Los números de acceso de secuencia se muestran entre paréntesis. En los nodos se muestran los valores de *bootstrap* iguales o superiores a 70% como porcentaje de 1000 réplicas. *Flexibacter flexilis* ATCC 23079^{T} se utilizó como *outgroup*. Barra, número de sustituciones por 100 posiciones nucleotídicas.

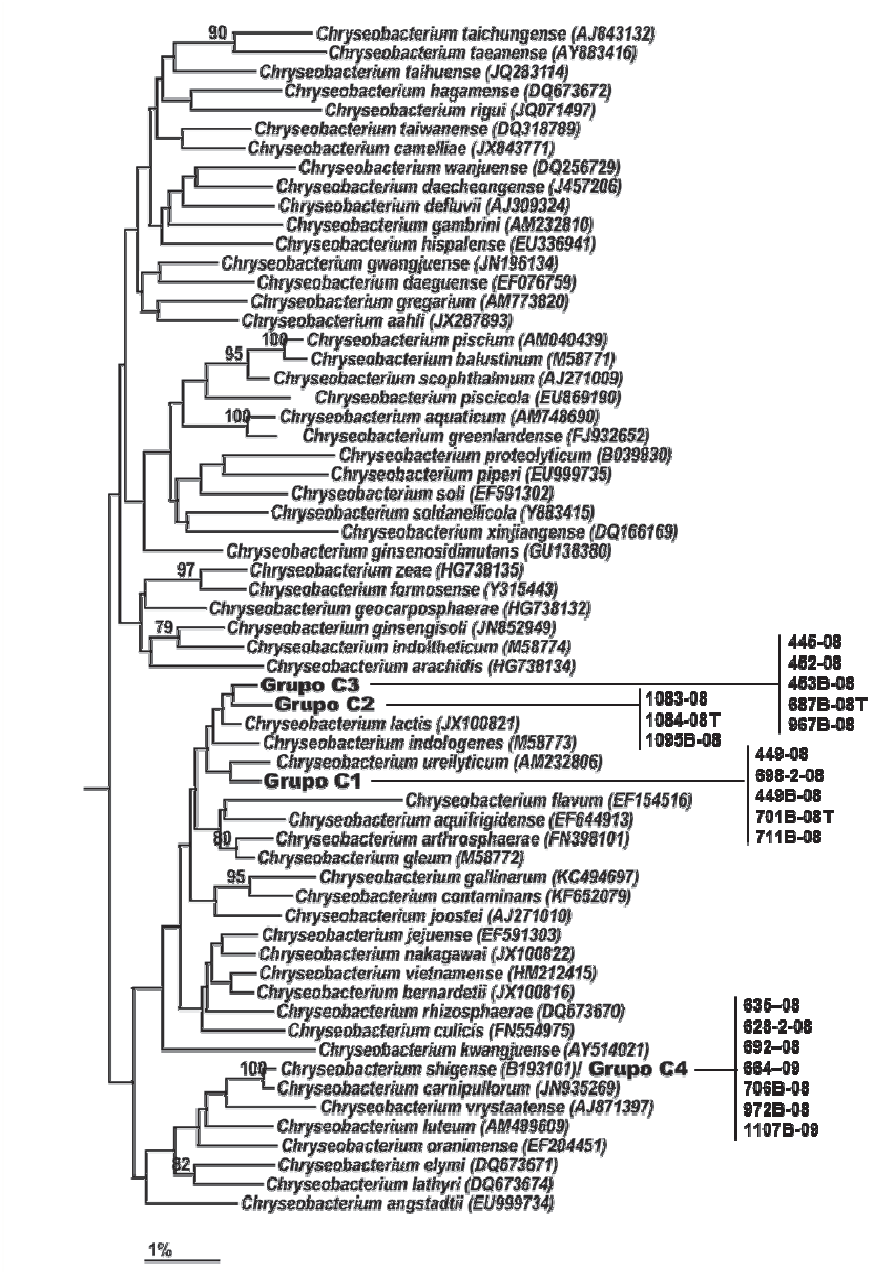

Figura 6. Árbol filogenético basado en la comparación de secuencias del gen 16S ARNr mediante el algoritmo *Neighbour Joining* en el que se muestra la posición de los aislados de trucha en relación con las especies filogenéticas más próximas del género *Chryseobacterium*. Los números de acceso de secuencia se muestran entre paréntesis. En los nodos se muestran los valores de *bootstrap* iguales o superiores a 70% como porcentaje de 1000 réplicas. *Flexibacter flexilis* ATCC 23079^{T} se utilizó como *outgroup*. Barra, número de sustituciones por 100 posiciones nucleotídicas.

Los porcentajes de homología del gen 16S ARNr de los aislados de trucha fueron en todos los casos superiores al 97% con las especies filogenéticamente más próximas. Estos porcentajes se muestran en la Tabla 7 para todos los grupos pertenecientes al género *Flavobacterium* y en la Tabla 8 para los grupos C1-C3 del género *Chryseobacterium*. Los aislados del grupo C4 presentaron porcentajes de homología del gen 16S ARNr con *C. shigense,* la especie más próxima filogenéticamente, del 99,2-99,8%. Las características de las colonias y la morfología observada en la tinción de Gram de estos aislados fueron idénticas a las observadas en la cepa tipo de *C. shigense* (GUM-Kaji^T; Shimomura y col., 2005). Además, la caracterización fenotípica de los aislados de este grupo mostró que la mayoría de las características eran consistentes con las indicadas en la descripción actual de esta especie (Shimomura y col., 2005), resultados que apoyaban la identificación obtenida mediante la secuencia de gen 16S ARNr. Basándonos en los datos de secuenciación de dicho gen, las características morfológicas y la caracterización fenotípica, los aislados del grupo C4 se identificaron como *C. shigense* (epígrafe 5.2, artículo 4). Esta especie fue aislada originalmente de una bebida de ácido láctico (Shimomura y col., 2005) y no hay publicaciones previas que relacionen a este microorganismo con peces, siendo esta la primera vez que se describe su aislamiento a partir de peces enfermos.

En los géneros *Flavobacterium* y *Chryseobacterium* especies diferentes pueden presentar homologías de hasta 99,0% en las secuencias del 16S ARNr (Bernardet 2010; Charimba y col., 2013; Subhash y col., 2013; Kämpfer y col., 2014a, b; Loch y Faisal, 2014b). Por este motivo realizamos estudios de hibridación ADN-ADN entre los aislados de los grupos F1-F7 y C1-C3 y las especies filogenéticamente más próximas. Los resultados mostraron porcentajes de hibridación que oscilaron en los grupos F entre 14,6 y 59,4% (Tabla 7) y en los grupos C entre 2,0 y 55,0% (Tabla 8) con las especies filogenéticamente más próximas a cada grupo, valores claramente inferiores al porcentaje del 70% de homología considerado como mínimo para asignar dos taxones a la misma especie (Wayne y col., 1987).

Tabla 7. Datos obtenidos del análisis de secuenciación del gen 16S ARNr (expresado en porcentaje de similitud) y del estudio de hibridación ADN-ADN (expresados en porcentaje de hibridación) de los diferentes grupos con las cepas tipo de las especies relacionadas filogenéticamente del género *Flavobacterium*

	Grupo F1		Grupo F2		Grupo F3		Grupo F4		Grupo F5		Grupo F6		Grupo F7	
Cepas tipo	**%Sim**	**%Hib**	**%Sim**	**%Hib**	**%Sim**	**%Hib**	**%Sim**	**%Hib**	**%Sim**	**%Hib**	**%Sim**	**%Hib**	**%Sim**	**%Hib**
F. chungangense	98,6	18,0	97,5	42,1	98,1	30,4	97,8	ND	97,6	ND	97,1	ND	98,2	38,4
F. frigidimaris	98,1	26,6	97,8	48,0	98,4	38,8	98,9	55,0	98,0	47,9	97,0	ND	98,6	59,4
F. hercynium	97,9	23,3	<97,0	ND	97,5	ND	97,7	ND	97,5	ND	97,7	ND	97,5	ND
F. aquidurense	97,8	19,5	97,9	38,0	98,6	14,6	98,5	28,2	98,2	28,9	<97,0	ND	98,7	27,5
F. pectinovorum	97,5	ND	97,9	44,0	97,9	ND	98,7	45,4	98,4	36,0	97,7	ND	98,1	54,5
F. hydatis	97,6	ND	97,6	21,0	97,2	ND	97,8	ND	98,0	30,9	98,8	30,9	97,6	ND
F. psychrolimnae	97,2	ND	<97,0	ND	97,5	ND	98,2	53,1	97,2	ND	<97,0	ND	97,2	ND
F. resistens	97,3	ND	<97,0	ND	97,5	ND	97,5	ND	<97,0	ND	<97,0	ND	97,9	ND
F. saccharophilum	97,5	ND	<97,0	ND	97,3	ND	98,1	37,7	98,3	19,6	97,4	ND	97,9	ND
F. granuli	97,1	ND	<97,0	ND	97,0	ND	97,4	ND	97,6	ND	98,3	46,8	97,4	ND
F. glaciei	97,5	ND	<97,0	ND	97,0	ND	97,0	ND	97,0	ND	97,4	ND	97,1	ND
F. succinicans	97,4	ND	<97,0	ND	<97,0	ND	97,6	ND	97,2	ND	97,3	ND	97,2	ND
F. micromati	97,1	ND	<97,0	ND	<97,0	ND	97,1	ND	<97,0	ND	<97,0	ND	97,4	ND
F. araucananum	<97,0	ND	97,6	26,1	98,5	33,4	98,5	38,1	97,7	ND	97,3	ND	98,2	55,8
F. hibernum	<97,0	ND	<97,0	ND	97,8	ND	97,5	ND	97,8	ND	97,6	ND	97,1	ND
F. tiangeerense	<97,0	ND	<97,0	ND	<97,0	ND	97,2	ND	97,8	ND	97,3	ND	<97,0	ND
F. limicola	<97,0	ND	<97,0	ND	<97,0	ND	97,3	ND	97,0	ND	97,0	ND	<97,0	ND
F. omnivorum	<97,0	ND	<97,0	ND	<97,0	ND	97,2	ND	97,3	ND	<97,0	ND	<97,0	ND
F. fryxellicola	<97,0	ND	<97,0	ND	<97,0	ND	97,3	ND	97,3	ND	<97,0	ND	<97,0	ND
F. xueshanense	<97,0	ND	<97,0	ND	<97,0	ND	<97,0	ND	97,9	ND	97,1	ND	<97,0	ND
F. chilense	<97,0	ND	<97,0	ND	<97,0	ND	<97,0	ND	97,6	ND	97,0	ND	97,8	ND
F. reichenbachii	<97,0	ND	<97,0	ND	<97,0	ND	<97,0	ND	97,0	ND	<97,0	ND	<97,0	ND
F. sinopsychrotolerans	<97,0	ND	<97,0	ND	<97,0	ND	<97,0	ND	<97,0	ND	<97,0	ND	97,0	ND
F. glycines	<97,0	ND	<97,0	ND	<97,0	ND	<97,0	ND	<97,0	ND	<97,0	ND	97,0	ND

%Sim, porcentaje de similitud; %Hib, porcentaje de hibridación; ND, No determinado

Tabla 8. Datos obtenidos del análisis de secuenciación del gen 16S ARNr (expresado en porcentaje de similitud) y del estudio de hibridación ADN-ADN (expresados en porcentaje de hibridación) de los diferentes grupos con las cepas tipo de las especies relacionadas filogenéticamente del género *Chryseobacterium*

	Grupo C1		Grupo C2		Grupo C3	
Cepa tipo	**% Sim**	**%Hib**	**%Sim**	**%Hib**	**%Sim**	**%Hib**
C. ureilyticum	99,1	53,0	98,0	39,0	98,6	47,0
C. joostei	98,6	52,0	97,0	16,0	97,8	35,0
C. jejuense	98,0	39,0	97,6	47,0	98,3	51,0
C. gleum	98,0	55,0	97,9	35,0	98,2	52,0
C. oranimense	98,0	34,0	<97,0	ND	97,5	45,0
C. indologenes	98,0	42,0	98,5	34,0	98,5	40,0
C. arthrosphaerae	97,9	20,0	97,7	28,0	98,0	31,0
C. shigense	97,5	36,0	97,2	2,0	97,6	45,0
C. aquifrigidense	97,5	34,0	97,1	19,0	97,2	42,0
C. luteum	97,3	20,0	<97,0	ND	97,1	22,0
C. culicis	97,2	20,0	<97,0	ND	97,5	50,0
C. lathyri	97,2	26,0	<97,0	ND	<97,0	ND
C. vrystaatense	97,0	2,0	<97,0	ND	<97,0	ND
C. hominis	<97,0	ND	97,5	30,0	97,7	33,0
C. rizhosphaerae	<97,0	ND	<97,0	ND	98,0	ND
C. scophthalmum	<97,0	ND	<97,0	ND	97,0	21,0

%Sim, porcentaje de similitud; %Hib, porcentaje de hibridación; ND, no determinado

Los resultados obtenidos en el estudio filogenético y de hibridación de ADN-ADN permitieron demostrar que los microorganismos estudiados se correspondían con siete nuevas especies dentro del género *Flavobacterium* y tres nuevas especies dentro del género *Chryseobacterium* para las cuales se propusieron los nombres específicos de *Flavobacterium oncorhynchi* sp. nov. (grupo F1), *Flavobacterium plurextorum* sp. nov. (grupo F2), *Flavobacterium tructae* sp. nov. (grupo F3), *Flavobacterium piscis* sp. nov. (grupo F4), *Flavobacterium collinsii* sp. nov. (grupo F5), *Flavobacterium branchiarum* sp. nov. (grupo F6), *Flavobacterium branchiicola* sp. nov. (grupo F7), *Chryseobacterium oncorhynchi* sp. nov. (grupo C1), *Chryseobacterium tructae* sp. nov. (grupo C2) y *Chryseobacterium viscerum* sp. nov. (grupo C3).

Con la finalidad de poder realizar la descripción formal de las nuevas especies, los estudios filogenéticos y de hibridación ADN-ADN se completaron con la determinación del contenido en G+C del ADN, el análisis quimiotaxonómico y la caracterización fenotípica que incluyó el estudio de las características morfológicas, fisiológicas, bioquímicas y nutricionales de los aislados.

El contenido en G+C del ADN varió para las especies del género *Flavobacterium* entre 33,0 y 36,2 mol% y para las especies del género *Chryseobacterium* entre 33,1 y 39,0 mol%, valores que están dentro del rango descrito para los géneros *Flavobacterium* y *Chryseobacterium* (Bernardet y Bowman, 2006; Bernardet y col., 2006).

El estudio de las características quimiotaxonómicas incluyó el análisis de ácidos grasos y menaquinonas en todas las nuevas especies y la determinación de los lípidos polares en seis de las nuevas especies incluidas en el género *Flavobacterium* (*F. oncorhynchi*, *F. tructae*, *F. piscis*, *F. collinsii*, *F. branchiarum* y *F. branchiicola*). Estas características, si bien no sirven para la identificación a nivel de especie, si son útiles para la discriminación a nivel de genero dentro de la familia *Flavobacteriaceae* (Bernardet 2010). Todas las especies presentaron MK-6 como principal quinona respiratoria y en algunas especies (*F. plurextorum*, *F. tructae*, *F. piscis, F. collinsii, F. branchiarum, F. branchiicola* y *C. oncorhynchi*) también se encontraron pequeñas cantidades de MK-5. En relación a los diferentes ácidos grasos, aunque existieron diferencias cuantitativas en el porcentaje de cada uno de los ácidos grados detectados entre las diferentes especies, todas ellas mostraron iso-$C_{15:0}$ como ácido graso mayoritario, observándose niveles de entre 19-33% para las nuevas especies

del género *Flavobacterium* y en torno al 33,7-40,9% para las del género *Chryseobacterium*. Respecto a los lípidos polares, la fosfatidiletanolamina fue el principal lípido polar en todas las especies estudiadas, aunque también se encontraron varios aminolípidos y lípidos no identificados. Todos los resultados quimiotaxonómicos obtenidos coinciden con los descritos para los miembros de los géneros *Flavobacterium* y *Chryseobacterium* (Vandamme y col., 1994a; Bernardet y col., 2002; Bernardet y Nakagawa, 2006; Bernardet y col., 2006) y confirman la adscripción de las nuevas especies a estos géneros basada en los estudios filogenéticos. Los resultados quimiotaxonómicos concretos concernientes a cada una de las nuevas especie se especifican en los epígrafes 5.1 (especies del género *Flavobacterium*) y 5.2 (especies del género *Chryseobacterium*).

En el estudio de las características fenotípicas se realizaron las pruebas recomendadas por Bernardet y col. (2002) para la descripción de nuevos miembros de la familia *Flavobacteriaceae.* Además, se estudiaron las pruebas contempladas en los sistemas comerciales API 20NE y API ZYM. Los resultados obtenidos en las pruebas realizadas para cada una de las nuevas especies se detallan en los correspondientes artículos donde se realiza su descripción formal (epígrafes 5.1 y 5.2). De igual forma, en la Tabla 9 se especifican las principales características que permiten diferenciar entre sí las nuevas especies de los géneros *Flavobacterium* y *Chryseobacterium.* Por ejemplo, las nuevas especies del género *Flavobacterium* podrían distinguirse por pruebas como la degradación de la L-tirosina (positiva para las especies *F. plurextorum*, *F. tructae*, *F. collinsii* y *F. branchiicola* y negativa para *F. oncorhynchi*, *F. piscis* y *F. branchiarum*), la degradación de la urea (únicamente positiva para *F. branchiicola*), la formación de pigmento difusible marrón en agar L-tirosina (observado solo en la especie *F. oncorhynchi*) y la actividad β-galactosidasa (ausente solamente en *F. branchiarum*). De igual forma, las nuevas especies del género *Chryseobacterium* pueden diferenciarse por pruebas como el crecimiento en medio BHI con 3% de NaCl (observado solamente en la especie *C. tructae*), la utilización de D-manitol como fuente de carbono y energía y la presencia de actividad N-acetil-beta-glucosaminidasa (ambas positivas únicamente para *C. viscerum*), la presencia de las enzimas tripsina y α-glucosidasa en *C. viscerum* y *C. oncorhynchi,* y la ausencia de actividad fosfoamidasa en *C. oncorhynchi.*

Tabla 9. Características diferenciales de las distintas especies de los géneros *Flavobacterium y Chryceobacterium* aisladas de peces.

Características	***F. oncorhynchi****	***F. plurextorum****	***F. tructae****	***F. piscis****	***F. collinsii****	***F. branchiarum****	***F. branchiicola****	***F. psychrophilum***	***F. branchiophilum***	***F. columnare***	***F. hydatis***	***F. jonshoniae***	***F. succinicans***	***F. araucananum***	***F. chilense***	***F. spartansii***	***C. viscerum****	***C. oncorhynchi****	***C. tructae****	***C. shigense****	***C. chaponense***	***C. piscicola***	***C. piscium***	***C. scophthalmum***
Gliding motility	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-	-	-	-	-	-	ND	-
Crecimiento en:																								
Agar Marino	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(+)	(+)	+	(+)	ND	-	ND	+
Agar Nutriente	+	+	+	+	+	+	+	+	-	V	+	+	+	+	+	+	+	+	+	+	+	+	+	ND
Agar Soja Tipticasa	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	ND	+
3% NaCl	-	-	-	-	-	-	-	-	-	-	-	-	ND	-	+	-	-	-	+	(+)	-	+	+	+
Crecimiento a:																								
25°C	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	ND	+	+	+	+	+	+	+	+
30°C	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	ND	+	+	+	+	+	-	+	+
37°C	-	-	-	-	-	-	-	-	-	+	-	-	ND	-	-	-	-	-	-	-	+	-	-	-
Degradación de:																								
Esculina	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	ND	+	+	+
Caseína	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+
DNA	-	-	-	-	-	-	-	ND	-	+	+	+	+	ND	ND	ND	-	-	-	-	-	(+)	(+)	+
Gelatina	-	-	+	+	-	-	-	+	+	+	+	+	ND	-	+	+	+	+	+	+	-	+	+	+
Almidón	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
L- tirosina	-	+	+	-	+	-	+	ND	+	-	+	+	-	+	+	ND	+	+	+	+	-	-	-	+
Urea	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	+	+	+	-	-	-	+	+
Absorción del rojo Congo	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	ND	-	ND	ND
Pigmento tipo flexirubina	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+
Pigmento difusible marrón en Agar L- tirosina	+	-	-	-	-	-	-	-	ND	ND	-	ND	-	-	ND	-	+	+	+	-	ND	ND	ND	ND
Producción de H_2S	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Tabla 9. Continuación

Características	*F. oncorhynchi**	*F. plurextorum**	*F. truncae**	*F. piscis**	*F. collinsii**	*F. branchiarum**	*F. branchiicola**	*F. psychrophilum*	*F. branchiophilum*	*F. columnare*	*F. hydatis*	*F. jonshoniae*	*F. succinicans*	*F. araucananum*	*F. chilense*	*F. spartansii*	*C. viscerum**	*C. oncorhynchi**	*C. truncae**	*C. shigense**	*C. chaponense*	*C. piscicola*	*C. piscium*	*C. scophthalmum*
Reducción de nitratos	+	+	+	+	-	-	+	-	-	ND	+	ND	D	+	ND	+	-	-	-	-	ND	-	+	-
Producción de indol	-	-	-	-	-	-	-	ND	-	ND	ND	ND	ND	-	-	-	+	+	+	+	-	-	+	-
Asimilación de:																								
L-arabinosa	+	+	-	+	-	-	+	ND	ND	ND	ND	ND	ND	+	+	-	-	-	-	-	-	ND	ND	ND
D-manitol	-	-	-	-	-	-	-	ND	ND	ND	ND	ND	ND	-		-	+	-	-	-	-	ND	ND	-
N-acetyl-glucosamina	+	+	+	+	+	+	+	ND	ND	ND	ND	ND	ND	+	+	+	-	-	-	-	-	ND	ND	ND
Malato	-	-	-	-	-	-	+	ND	ND	ND	ND	ND	ND	-	+	-	-	-	-	-	ND	ND	ND	ND
Citrato	-	-	+	-	+	+	-	ND	ND	ND	ND	ND	ND	-	-	+	+	+	+	+	-	ND	ND	-
Producción de:																								
Ester lipasa	-	-	-	-	-	-	-	ND	+	ND	ND	ND	ND	(+)	ND	+	+	+	+	-	ND	+	ND	ND
Valina arilamidasa	-	-	+	-	-	+	+	ND	+	ND	ND	ND	ND	+	ND	+	+	+	+	+	ND	+	ND	ND
Tripsina	-	-	-	-	-	-	-	ND	-	ND	ND	ND	ND	-	ND	-	+	+	-	+	ND	-	ND	ND
α-Galactosidasa	-	-	-	+	-	-	-	ND	-	ND	ND	ND	ND	-	ND	+	-	-	-	-	ND	-	ND	ND
α-Glucosidasa	+	+	-	+	-	-	-	ND	-	ND	ND	ND	ND	+	ND	+	+	+	-	-	+	+	ND	ND
N-acetil-beta-glucosanamidasa	+	+	-	+	-	+	+	ND	-	ND	ND	ND	ND	(+)	ND	+	+	-	-	-	ND	+	ND	+
β-Galactosidasa	+	+	+	+	+	-	+	-	-	-	+	+	+	+	ND	V	-	-	-	-	-	-	ND	ND

+, Reacción positiva; -, reacción negativa; (+), reacción positiva débil; D, dudoso; ND, no definido; V, variable; *especies identificadas en este trabajo

Los microorganismos que se aíslan de un amplio rango de ambientes, como es el caso de los géneros *Flavobacterium* y *Chryseobacterium*, suelen ser genéticamente heterogéneos (Bernardet y col., 2006; Martín y col., 2007). Sin embargo, aislados recuperados de alevines diferentes y procedentes de diferentes episodios clínicos que tuvieron lugar en diferentes periodos de tiempo (Tabla 6), mostraron patrones indistinguibles cuando se caracterizaron por PFGE indicando que se trataban de una misma cepa. Este es el caso de las especies *F. oncorhynchi* y *C. oncorhynchi* (epígrafe 5.1, artículo 1, Fig. 3 y epígrafe 5.2, artículo 1, Fig. 2, respectivamente). Algo similar ocurrió con los aislados de *C. shigense* cuando se tipificaron mediante la técnica RAPD, ya que mostraron un único perfil molecular a pesar de haber sido aislados durante cinco episodios clínicos diferentes (epígrafe 5.2, artículo 4, Fig. 2). Además, las especies de los géneros *Flavobacterium* y *Cryseobacterium* que se describen en este trabajo, como hemos indicado en párrafos anteriores, se aislaron mayoritariamente de alevines enfermos y en algunos casos en cultivo puro a partir de órganos internos (Tabla 6). Estos datos, junto a los resultados obtenidos tras la caracterización genética mediante PFGE o RAPD permiten sugerir una significación clínica para algunas de las especies descritas. En este mismo sentido, en un estudio reciente en el que se estudió la diversidad de flavobacterias aisladas de diferentes especies de peces con distintas patologías en el estado de Michigan, EEUU (Loch y col., 2013), se aislaron, junto a los patógenos clásicos *F. psychrophilum* y *F. columnare,* tres de las especies descritas en el presente trabajo de Tesis Doctoral (*F. oncorhynchi, C. viscerum* y *C. shigense*). *C. viscerum* y *C. shigense* se aislaron de las branquias de diferentes especies de peces y *F. oncorhynchi* se aisló de los riñones de varias especies de peces enfermos que presentaban diferentes signos clínicos, como congestión de las aletas, exoftalmia unilateral, palidez de bazo, hígado y riñón, y en ocasiones necrosis epitelial e hiperplasia de las branquias. Estos datos apoyarían la posible significación clínica de algunas de las especies descritas en este trabajo de investigación.

Con la excepción de las especies *F. branchiophilum*, *F. columnare* y *F. psychrophilum* la mayoría de los miembros de los géneros *Flavobacterium* y *Chryseobacterium* suelen considerarse patógenos oportunistas capaces de ocasionar un proceso clínico bajo determinadas situaciones de estrés, como condiciones pobres de cultivo o trastornos ambientales, u otras circunstancias predisponentes tales como coinfecciones con otras bacterias o virus (Bernardet y Nakagawa, 2006). En este sentido, estudios de infección

experimental realizados con *F. chilense* y *F. spartansii*, dos nuevas especies del género *Flavobacterium* aisladas de truchas enfermas (Tabla 3, epígrafe 3.2.1) sugieren asimismo que ambas especies serían patógenos oportunistas al no ser capaces de producir mortalidad ni signos clínicos en alevines de trucha infectados experimentalmente (Avendaño-Herrera y col., 2014). En nuestro estudio, los episodios clínicos ocurrieron generalmente después del transporte de los alevines de trucha arco iris desde la incubadora a la piscifactoría o cuando los tanques de transporte tenían una elevada densidades de alevines. Estas circunstancias representan condiciones estresantes para los peces (Georgiadis y col., 2001) que podrían haber aumentado la susceptibilidad de los alevines de trucha a la infección. Estudios preliminares de patogenicidad experimental que realizamos con la cepa tipo de *F. oncorhynchi* (datos no publicados) apuntan al posible carácter de patógeno oportunista de esta especie. Así, esta cepa solo fue capaz de ocasionar lesiones y mortalidad en alevines de trucha tras someterles a una situación de estrés manual severo. No obstante, el papel de estas especies como patógenos de peces deberá confirmarse mediante ensayos exhaustivos de infecciones experimentales, estudios que están fuera de los objetivos del presente trabajo de investigación.

Como ya hemos mencionado, en el género *Flavobacterium* se incluyen algunas especies que son reconocidas como importantes patógenos de peces (*F. branchiophilum*, *F. columnare* y *F. psychrophilum*) y otras que se han aislado de peces enfermos (Ostland y col., 1994; Barnes y Brown, 2011; Kampfer y col., 2012; Declercq y col., 2013; Loch y Faisal, 2014b). Por el contrario, dentro del género *Chryseobacterium* no hay ninguna especie definida como patógena para peces, si bien en los últimos años se han aislado varias especies de muestras clínicas de peces enfermos (Mudarris y col., 1994; De Beer y col., 2006; Flemming y col., 2007; Ilardi y col., 2009). Por tanto, independientemente de que muchas de estas especies no puedan considerarse claramente como patógenos de peces, resulta conveniente conocer aquellas características fenotípicas que puedan facilitar su identificación. En este sentido, en la Tabla 9 se resumen las principales características fenotípicas de las especies de los géneros *Flavobacterium* y *Chryseobacterium* que se han aislado hasta el momento de peces enfermos y que pueden ser útiles para su diferenciación en el diagnóstico microbiológico realizado en el laboratorio.

El presente estudio, donde se describe el aislamiento de 10 nuevas especies de los géneros *Flavobacterium* y *Chryseobacterium* y se describe por primera vez el aislamiento de la especie *C. shigense* a partir peces enfermos, ha permitido tener un mejor conocimiento de la diversidad de especies de ambos géneros relacionadas con infecciones en peces. Además, estos resultados junto al aislamiento creciente de especies de ambos géneros a partir de diferentes muestras clínicas (Pacha y Porter, 1968; Rintamaki-Kinnunen y col., 1997; Tiirola y col., 2002; Bernardet y col., 2005; Flemming y col., 2007; Ilardi y Avendaño-Herrera, 2008; Loch y col., 2013) ponen de manifiesto el papel de estos microorganismos como potenciales patógenos de peces.

Conclusiones

7 Conclusiones

1. Las bacterias aisladas de alevines y huevos embrionados de trucha arcoíris se relacionaron filogenéticamente con los géneros *Flavobacterium* y *Chryseobacterium*.

2. El estudio taxonómico polifásico de las flavobacterias estudiadas permitió la descripción de 7 nuevas especies del género *Flavobacterium (Flavobacterium oncorhynchi*, *Flavobacterium plurextorum*, *Flavobacterium tructae, Flavobacterium piscis, Flavobacterium collinsii, Flavobacterium branchiarum* y *Flavobacterium branchiicola*) y 3 nuevas especies del género *Chryseobacterium* (*Chryseobacterium oncorhynchi*, *Chryseobacterium tructae* y *Chryseobacterium viscerum)*.

3. Las especies *Flavobacterium plurextorum* y *Flavobacterium collinsii* incluyeron aislados de alevines y huevos embrionados.

4. Se describe por primera vez el aislamiento de *Chryseobacterium shigense* a partir de muestras clínicas de peces.

5. Este estudio pone de manifiesto la diversidad de microrganismos de los géneros *Flavobacterium y Chryseobacterium* relacionados con la producción piscícola de trucha arcoíris en España.

6. Los resultados de caracterización molecular de los aislados de *Flavobacterium oncorhynchi*, *Chryseobacterium oncorhynchi* y *Chryseobacterium shigense* sugieren una posible implicación de estas especies en el síndrome del alevín de la trucha arcoíris, si bien su papel como patógenos de peces deberá confirmarse mediante estudios posteriores.

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8 Bibliografía

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